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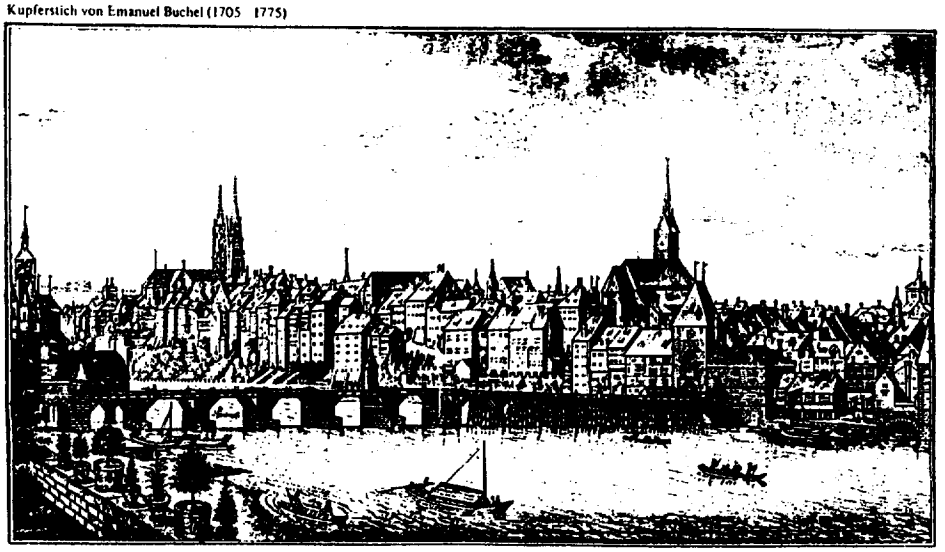
Abstracts

Kupferstich von Emanuel Buchel (1705 1775)



PROSPECT DER RHEINBRÜCKE ZU BASEL VON SEITEN DER KLEINEN STADT

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PLENARY SESSIONS (S 1 - 32)

Cytochromes P450

C. Roland Wolf

Imperial Cancer Research Fund, Molecular Pharmacology Group, Hugh Robson Building, George Square, Edinburgh EH8 9XD, U.K.

The cytochrome P450 multigene family plays a central role in the metabolism and disposition of drugs, chemical toxins and carcinogens. In addition to catalyzing the detoxification of many of these compounds this enzyme system can ironically lead to the conversion of certain of them to their ultimately toxic or carcinogenic products.

The application of recombinant DNA techniques to the study of the P450 system has vastly increased our knowledge of the structure, function and regulation of these proteins. In mammals it is clear that the P450 system has diverged into a series of multigene families (or subfamilies) scattered through the genome. The number of members found in each of these families varies from one to as many as eight or ten.

In humans hepatic cytochrome P450 isozyme content is subject to large individual variation. It appears that this variability is determined by either genetic, environmental or hormonal factors. Significant progress has been made in understanding the molecular basis of the genetic polymorphism associated with the P450IID gene family however little progress has been made on the other well defined polymorphism in the P450IIC subfamily.

Animal studies have given us important clues on the environmental and hormonal factors which regulate cytochrome P450 expression. Pharmacological evidence indicates foreign compound regulation of P450 expression in man is similar to that found in rodents. The role of hormones in the regulation of P450 expression in man however is unclear.

In this presentation current progress in these research areas will be discussed and progress on the relationship between the expression of specific cytochrome P450 isozymes and pharmacological and toxicological response will be described.

GLUTATHIONE S-TRANSFERASES

Lesley I McLellan, Department of Clinical Chemistry, University of Edinburgh, U.K.

Glutathione S-transferases (GST) are a supergene family of isoenzymes which can act to protect cellular macromolecules from attack by reactive electrophiles. Protection may be mediated catalytically, where glutathione is conjugated with electrophilic chemicals and carcinogens; some GST also possess glutathione peroxidase activity towards organic peroxides, representing a further major catalytic mechanism for detoxication. Alternatively, the GST may interact with many lipophilic compounds which are not substrates by binding, either covalently or non-covalently.

At least three classes of cytosolic GST exist in mammals. These classes are named Alpha, Mu and Pi. They consist of dimeric proteins with apparent subunit Mr values of 24 800-27 500. A membrane-bound GST isoenzyme has also been identified in microsomes and mitochondrial membranes; by contrast to the cytosolic GST, this is a trimeric protein with subunit Mr of 17 300.

Many model substrates have been described for GST, some of which are diagnostic for a particular subunit type. There are also several naturally occurring GST substrates; these include 4-hydroxyalk-2-enals and cholesterol α-oxide, both toxic products of lipid peroxidation, and aflatoxin-8,9-epoxide, a carcinogenic metabolite of aflatoxin B_1. Physiological functions of GST with endogenous substrates include steroid isomerase and leukotriene C_4 synthetase activity. Many environmental pollutants are potential substrates for GST, although conjugation with glutathione may not always result in detoxication; for example, metabolism of the glutathione-conjugate of hexachloro-1,3-butadiene results in the formation of a potent nephrotoxin.

Mechanisms for the control of expression of GST are being extensively studied, partly because of the postulated involvement of these enzymes in drug-resistance. Many GST are readily inducible by xenobiotics such as phenobarbital, 3-methylcholanthrene and the antioxidants butylated hydroxyanisole and butylated hydroxytoluene. Butylated hydroxyanisole-mediated elevation of GST levels correlates with resistance to chemically-induced carcinogenesis in mice. Glutathione S-transferases are also subject to tissue-specific and sex-specific control. In mammals the highest concentrations of cytosolic and microsomal GST are in the liver, but they are present in significant amounts in extra-hepatic organs. Sex-differences in GST-expression exist in mice and rats, particularly in liver and kidney, but the physiological implications are not known.

Glucuronosyl Transferases: Reactions and Isoenzymes

K.W.Bock, P. Münzel, D. Schrenk and M. El Mouelhi
Institute of Toxicology, Wilhelmstraße 56, 7400 Tübingen, FRG

Glucuronide formation, catalysed by glucuronosyl transferases (GTs), represents a major process through which numerous nucleophilic endo- and xenobiotics are converted to more water-soluble and readily excretable products. A broad range of functional groups are used as acceptors of glucuronic acid, leading to O- (ether and ester), N-, S-, Se- and C-glucuronides. The role of GTs in detoxication can be appreciated best in the context of other drug metabolizing enzymes, such as P450s and glutathione transferases.

Ester and N-glucuronides are unstable above and below pH 7.4, respectively. The resulting aglycones may initiate toxic reactions. Moreover, glucuronides are hydrolysed by bacterial β-glucuronidases in the intestine. In this way glucuronides represent stable transport forms for toxins and may initiate toxicity after non-enzymatic or enzymatic liberation of toxins at the target site (urinary bladder or colon, respectively).

Recent advances in enzyme purification and in molecular biological approaches have established that glucuronosyl transferases represent a large enzyme family consisting of isozymes with distinct but overlapping substrate specificity. Marked differences in the tissue distribution of GT isoenzymes have been noted. Moreover, both differences and similarities of GTs have been found in different species, such as rat and man. There is still a need for selective functional and molecular probes for different GT isoenzymes. Nevertheless, it is conceivable that most drugs may be overlapping substrates for several isoenzymes. (S)- and (R)-naproxen, which form ester glucuronides, are stereoselectively conjugated by at least 3 different rat liver isoenzymes. One rat liver GT isoenzyme (phenol GT or GT-1) will be emphasized which is co-regulated with cytochrome P450IA1 by the Ah or dioxin receptor. The latter example will serve to discuss current problems of GT nomenclature and of the significance of GT assays.

MOLECULAR MECHANISMS OF REGULATION OF DRUG METABOLISM

G. Gordon Gibson

University of Surrey, Biochemistry Department, Molecular Toxicology Group, Guildford, Surrey GU2 5XH, England, U.K.

Regulation of drug metabolism is a complex and multi-factorial process and much effort has been expended in recent years in examining the molecular mechanisms of this phenomenon. For example, substrate, cofactor and oxygen supply will critically influence the rate of drug biotransformation, but it should be emphasised that most of our knowledge in this area is derived from studies on the regulation of the drug metabolising enzymes themselves, particularly the cytochrome P450 superfamily.

Apart from the intrinsic kinetic interactions of a drug and its cognate enzyme, the absolute amount of enzyme is an important determinant of drug biotransformation and the steady state enzyme concentration is a balance between enzyme synthesis and degradation. For example, haem synthesis and supply to form catalytically-competent holo-cytochrome P450 has been extensively studied and xenobiotics can modulate haem supply by several molecular mechanisms including phenobarbital induction of haem synthesis and either direct or biotransformation-dependent inhibition of haem synthesis or function via the mechanisms of haem alkylation or ligation, both resulting in inhibition of enzyme activity.

As a result of the application of molecular biology techniques, enzyme regulation by xenobiotics is an area of active research and steady state enzyme concentrations can be induced by transcriptional activation of the corresponding genes, mRNA stabilisation and post-translational regulation. For example, cytochrome P450IIE1 combines more than one of these regulatory mechanisms dependent on the nature of the inductive stimulus. In addition, the cytochrome P450 IA sub-family is regulated at the gene level by a complex combination of negative and positive control elements identified in the 5', upstream region of the gene. Recent studies on the cytochrome P450 family have demonstrated, via domain exchange technology, that certain regions of the enzymes are important for catalytic activity, presumably dictated by the substrate binding site, and this powerful new approach to enzyme structure and function has already yielded chimaeric constructs expressing low and high activity forms of the drug metabolising enzymes.

Physiological and pathophysiological regulation of the prevailing drug metabolising enzyme complement has significantly contributed to our knowledge of regulation of drug biotransformation, as witnessed by the extensive studies on hormone regulation. Furthermore, pathophysiological control of drug biotransformation such as occurs in diabetes, has been rationalised by a complex process of insulin-dependent, transcriptional gene regulation, as mediated through insulin-dependent perturbation of intermediary metabolism. Other constitutive chemical messengers such as the interferons, down-regulate drug metabolism by depression of the steady-state level of cytochrome P450mRNAs, although whether this results from an interferon influence on gene transcription or mRNA stability is not clear at present.

Accordingly, regulation of drug metabolism is a complex event, critically dependent on both external and internal factors which may be ultimately manifested as either an increase or decrease in drug biotransformation capacity.

TISSUE SPECIFIC EXPRESSION OF CYTOCHROMES P-450

Ph. BEAUNE, I. DE WAZIERS

INSERM U 75 - 156 rue de Vaugirard 75730 PARIS CEDEX 15

Cytochrome P-450 are a superfamily of enzymes involved in xenobiotic metabolism. Numerous enzymes were described in mammals and also in man. Most of the enzymes are mainly expressed in liver which is the main organ for xenobiotic metabolism. However most other tissues, "extrahepatic tissues", contains measurable amount of Cytochrome P-450 and other xenobiotic metabolizing enzymes such as epoxide hydrolase, glutathione-S-transferases or UDPG-transferase. Nevertheless, the quantity and the distribution of these different enzymes or isoenzymes vary from tissue to tissue. These variations play a key role in tissue specificity of xenobiotic metabolism and therefore on xenobiotic toxicity and local pharmacological effects.In this presentation we will focus on the tissue distribution of xenobiotic metabolizing enzymes in man. In a recent study we have shown that, in man, oesophagus, small intestine, colon and kidney contained cytochromes P-450 in low amounts as compared to liver. The pattern of the different P-450 enzymes was different from tissue to tissue. P-450 III A was the more ubiquitous P-450.

The pharmacological and toxicological implications of this distribution will be discussed.

HORMONAL CONTROL OF DRUG METABOLISM

David J. Waxman

Dept. of Biological Chemistry and Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, U.S.A.

Hepatic cytochrome P450 enzymes play a key role in the oxidative metabolism of numerous lipophilic drugs, and are a major determinant of the activity and in vivo responses to many pharmacologic agents. In the rat, and other species as well, constitutively expressed drug-metabolizing P450 enzymes can be regulated by multiple hormones. The widely studied inductive and suppressive effects that many drugs have on P450 enzyme levels can also be influenced by endogenous hormone levels. In some cases these effects appear to involve a perturbation of hormonally regulated P450 gene expression. Outlined below are the key roles that have been described for several classes of pituitary-dependent hormones on the basis of studies carried out in rat liver.

(a) Androgens play a crucial role during the neonatal period in programming (imprinting) the expression of several adult male-specific hepatic P450 enzymes [forms RLM2 (IIA2), 2c (IIC11), g (IIC13) and 2a (IIIA2). The action of androgens on these P450s does not occur directly at the liver, but is primarily mediated by the hypothalamo-pituitary axis and its control of hepatic gene expression.

(b) Growth hormone (GH) - The sexually differentiated pattern of pituitary GH secretion has a major impact on hepatic P450 levels. The pulsatile profile of plasma GH that is characteristic of adult male rats supports expression of several male-specific P450s. In contrast, the more continuous plasma GH profile of adult female rats stimulates expression of female-predominant hepatic drug metabolizing enzymes (eg., P450 2d (IIC12)), while it concomitantly suppresses expression of male-specific P450 enzymes.

(c) Thyroid hormones regulate P450-dependent hepatic drug metabolism by multiple mechanisms. These include: (1) maintenance of the flavoenzyme NADPH P450 reductase, which is an obligatory and rate-limiting component for all microsomal P450 reactions; (2) suppression of the male-specific steroid 6β-hydroxylase P450 2a (IIIA2), by acting in concert with the suppressive effects of continuous GH on this hepatic enzyme; (3) direct, as well as indirect stimulation of the expression of several female-predominant hepatic drug-metabolizing P450s.

Several useful models of endocrine depletion are available for studying the contributions of these hormones to hepatic drug metabolism. These include gonadectomy, hypophysectomy, hypothyroidism induced by the anti-thyroid drug methimazole, and destruction of the arcuate nucleus of the hypothalamus by neonatal administration of monosodium glutamate (MSG). Application of these treatments, in combination with suitable hormone replacement regimens, has established that the effects of pituitary-dependent hormones on hepatic drug metabolism occur largely, if not exclusively, by pretranslational mechanisms. In contrast, hormones such as glucagon may contribute to the short term regulation of hepatic P450 activity by post-translational mechanisms, such as P450 protein phosphorylation, which has been shown to occur both in isolated hepatocytes and in vivo.

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P450 GENE SUPERFAMILY: EVOLUTION AND REGULATION

Daniel W. Nebert, University of Cincinnati Medical Center, Cincinnati OH 45267-0056 USA

A nomenclature system for the P450 superfamily based on evolution has been established during the past 5 years. The P450 genes are named with the root "CYP," denoting cytochrome P450. Gene families are designated by numbers; subfamilies (when they exist) are represented by letters, followed by a number for the individual gene. A protein encoded by a gene in one family usually exhibits <40% amino acid sequence similarity. Mammalian genes within the same subfamily encode proteins >59% identical. This somewhat arbitrary classification has proven to be very useful, since all genes in any subfamily so far examined lie within clusters in the mouse and human genomes.

The P450 superfamily presently comprises 21 gene families, ten of which exist in all mammals. Current estimates of the total number of functional P450 genes in any given mammalian species range between at least 60 and perhaps more than 200. Each functional gene almost always encodes a unique enzyme. The superfamily has undergone divergent evolution, and the ancestral gene is probably more than 2.5 billion years old. The recent "burst" in new P450 genes, particularly in the CYP2 family during the past 800 million years, is likely to be the result of "animal-plant warfare." Due to the presence or absence of a particular P450 gene in one species but not the other, there might be problems in extrapolating toxicity data or cancer data from rodent to human. Increases in the P450 gene product (enzyme induction) almost always reflect an elevated rate in gene transcription, although there are several noteworthy exceptions.

The determination of catalytic activity might not be helpful in assigning orthologous genes across species. Negishi and coworkers have shown that a single amino acid difference—in a peptide of about 500 residues—can be critical in changing the catalytic activity from "coumarin 7-hydroxylase" to "testosterone 15α-hydroxylase." These results indicate that a particular ancestral gene 20 million years ago, for example, could have undergone a single nucleotide substitution in rat but not in mouse 10 million years ago, leading to a single amino acid change, such that the orthologous gene in the rat and mouse would exhibit entirely different catalytic activities. [The rat and mouse species are believed to have separated from one another about 17 million years ago.]

Divergence of the P450 genes has been found to be markedly nonlinear. As a case in point, the human and chicken CYP17 genes appear to have diverged more than twice as long ago as the human and chicken CYP19 genes, yet the original CYP17 gene and CYP19 gene undoubtedly existed long before the predecessors of mammals and birds split. This discrepancy points out the difficulties with any computer program that attempts to define a phylogenetic tree from a combination of fossil data and molecular biology data. Another consideration is the real possibility that the CYP17 and the CYP19 genes have not diverged at the same rate.

Gene conversion events can re-establish 100% similarity in a portion (or all) of a gene that had been considerably diverged from its neighbor. There are numerous examples of gene conversion events among homologous genes in various P450 gene subfamilies, indicating that gene conversion is a major cause for the nonlinearity of P450 gene evolution. Gene conversion events thus create the biggest problem with any computer-alignment program that cannot take this into account.

A large number of allelic variants have been identified in the human and rodent. We have arbitrarily assigned proteins derived from two alleles of the same gene as having "≤3% divergence," unless functional differences (catalytic activities) have been demonstrated. This would mean fewer than 15 amino acid differences among a 500-residue P450 protein; however, there are two cases so far in which this arbitrary cut-off does not hold: the rat b and e proteins are 97.4% identical and the mouse coumarin 7-hydroxylase and 15α-hydroxylase differ at only 11 residues. In other cases as well, it has been difficult to distinguish allelic variants from products derived from two distinct genes. More extensive gene mapping and cloning studies in the future should help clarify some of this confusion. One consequence of human P450 gene evolution is polymorphism in drug metabolism, leading to marked differences in individual risk to the toxic and carcinogenic effects of drugs and other environmental chemicals.

FLAVIN-CONTAINING MONOOXYGENASES: CATALYTIC ACTIVITY AND SUBSTRATE SPECIFICITY

L A Damani

Chelsea Department of Pharmacy, King's College London, University of London, Manresa Road, London SW3 6LX, U.K.

The flavin-containing monooxygenase(FMO) is listed in Enzyme Nomenclature (Webb, 1984) as "Dimethylaniline monooxygenase (N-oxide forming, EC.1.14.13.8)". It has been described as an amine oxidase and refered to as the Ziegler's Enzyme, after Ziegler and co-workers who first purified this enzyme to homogeneity from pig liver microsomes. This enzyme and the cytochrome P-450-dependent monooxygenase system are the major enzymes catalyzing the oxidation of lipophilic foreign nucleophilic nitrogen-, sulphur-, and phosphorus-containing organic compounds. As in the case of P-450 monooxygenases, it is now clear that there are probably several microsomal flavin-containing monooxygenases, with overlapping substrate specificiies, and that the relative proportions of these isozymes may vary in different tissues within and between species.

Metabolic N-, S-, and P-oxygenations represent important routes of metabolism for a large number of drugs and other foreign compounds. Therefore there has been an interest in delineating the relative contributions of P-450 and FMO to such reactions. Any changes in the activity of enzymes involved in specific oxygenations through , environmental, physiological or genetic factors, would have an effect on disposition, and an effect ultimately on drug pharmacology and/or toxicology. Qualitative predictions as to which enzyme may be utilized for a specific N-, S-, or P-oxygenation are possible, based on mechanistic and physicochemical considerations. In this lecture, the presenter will examine the catalytic activity of the FMO towards various nitrogen-, sulphur-, and phosphrous-containing compounds, and compare these with similar P-450 mediated reactions, with particular emphasis on the stereochemical outcome of reactions catalyzed by the monooxygenases. In addition, the status of both these monooxygenases in isolated cell systems(hepatocytes and epithelial cells from the gastrointestinal tract) will also be presented, together with a description of new sensitive methods for the estimation of FMO activity in cell systems. Approaches to monitoring the activity of this enzyme *in vivo* will also be described.

ALDO-KETO REDUCTASES

Bendicht Wermuth

University of Berne, Department of Clinical Chemistry, Inselspital, CH-3010 Berne, Switzerland

The aldo-keto reductases comprise a family of cytosolic enzymes with similar physical and chemical properties which catalyze the NADPH-dependent reduction of aldehydes and ketones to the corresponding alcohols. Members of the family are present in all higher animals and most tissues so far investigated. On the basis of a preferential, but not mutually exclusive, substrate specificity they are divided into aldehyde reductases, represented by aldehyde reductase (EC 1.1.1.2) and aldose reductase (EC 1.1.1.21), and ketone reductases, represented by carbonyl reductase (EC 1.1.1.184). Aldehyde reductases preferentially catalyze the reduction of aromatic and aliphatic α-hydroxyaldehydes, including sugar aldehydes and aldehydes derived from the biogenic amines and corticosteroid hormones. With the exception of negatively charged aldehydes which are the preferred substrates of aldehyde reductase, aldose reductase exhibits Km values 1 to 2 orders of magnitude lower than those of aldehyde reductase. Carbonyl reductase most efficiently catalyzes the reduction of quinones, e.g. quinones derived from polycyclic aromatic hydrocarbons, and aromatic aldehydes. The enzyme also exhibits low but significant activity with prostaglandins and 3-ketosteroids. In spite of the many physiological and xenobiotic carbonyl compounds that are substrates of the aldo-keto reductases, and although their ubiquitous distribution suggests them to perform fundamental roles, no vital metabolic function could be attributed to any of the enzymes as yet.

Aldo-keto reductases characteristically consist of a single polypeptide chain containing no carbohydrate. The average chain length of aldehyde reductases is 320 amino acids (Mr 35,000), that of carbonyl reductase 280 amino acids (Mr 30,000). Multiple molecular forms, most probably of posttranslational origin and of unknown physiological significance, exist of aldose and carbonyl reductase. The complete protein and/or cDNA sequences of all three human aldo-keto reductases as well as of aldose reductase from rat and ox have been established. The results indicate structural homology between aldehyde and aldose reductase of about 50 per cent and between aldose reductases from different species of about 80 per cent. Moreover, the two enzymes are members of a protein superfamily which also includes bovine prostaglandin F synthase, 2,5-diketo-D-gluconate reductase from Corynebacterium, a developmentally regulated protein from Leishmania and frog lens ρ-crystallin . Carbonyl reductase shows some characteristics of the "short-chain" dehydrogenases although no obvious homology to any of the proteins that have so far been sequenced is detectable.

ESTERASES AND HYDROLASES

C.H. Walker

Department of Biochemistry & Physiology, The University, P.O. Box 228, Whiteknights, Reading, RG6 2AJ, U.K.

A wide range of xenobiotic esters, many of them lipophilic in character, are readily hydrolysed by esterases located in membranes, cytosol and plasma of vertebrates. In the classification of Enzymes of the International Union of Biochemistry, esterases (EC 3.1) are defined as one of eleven major subdivisions of hydrolases. However, some esterases have very wide substrate specificities, and can hydrolyse non-ester bonds. Thus certain esterases can hydrolyse peptide bonds (EC 3.4), carbon-nitrogen bonds other than peptide
(EC 3.5) and carbon-halide bonds (EC 3.8). The following classes of enzymes will be discussed, emphasising their properties and their significance in regard to selective toxicity:-

1) Carboxyl esterases (EC 3.1.1.1). A recent genetic classification identified 11 distinct esterases of this type in the mouse. Four different forms have been purified from rat liver microsomes. Each hydrolyses a wide range of exogenous and endogenous substrates. There are marked species differences in these esterases which may be important in determining selective toxicity.

2) Aryl esterases (EC 3.1.1.2). Hydrolyse phenyl acetate and are insensitive to inhibition by organophosphates. Some forms do not hydrolyse organophoshorus substrates such as paraoxon.

3) Phosphoric triester hydrolases (EC 3.1.8). Found in HDL fraction of mammalian serum, and liver microsomes. These enzymes are Ca^{2+}-dependent and can hydrolyse paraoxon and other organophosphates. The absence or low activity of these enzymes in avian serum, contributes to the sensitivity of birds to certain organophosphorus insecticides.

4) DFP-ase (EC 3.8.2.1), and similar hydrolases act upon carbon-halide and carbon-cyanide bonds and have an important role in the detoxication of nerve agents such as Soman and Tabun.

REACTIONS, ENZYMES AND REGULATION: DEVELOPMENTAL REGULATION

Anders Rane
Division of Clinical Pharmacology, Academic Hospital, S-751 85 Uppsala, Sweden

Drug oxidation reactions are not developed at significant rates prior to birth in subprimate experimental animals. In contrast the human fetal liver catalyzes the oxidation of several drugs and endogenous substrates but the metabolic rates are generally low, between 5 and 30 % of adult values.

However, one enzyme reaction with ethylmorphine (EM) as substrate was recently shown to be about as active in human fetal as in human adult liver (Ladona et al, Biochem, Pharmacol., 38, 3147, 1989). This EM N-demethylase is detected by antibodies towards PCN-induced rat liver cytochrome P-450 in immunoblotting and is probably ralated to a human fetal erythromycin N-demethylase which is identified by antibodies against rat cytochrome P-450 p (Wrighton et al, Biochem. Pharmacol., 37, 3053, 1988). It has probably an important physiological role in the metabolism of steroids during fetal development since it catalyzes e.g.the 16 α - hydroxylation of dehydroepiandrosterone. Ample evidence has been presented that this enzyme belongs to the cytochrome P-450 IIIA gene subfamily. The identity and functions of the fetal P-450 IIIA enzyme(s) is subject of current investigations in various laboratories. Substrate specificity studies as well as molecular biology approaches are applied. There is a close relation between the fetal enzyme and the adult P-450 IIIA3 and IIIA4 enzymes as judged by comparisons of partial amino acid sequiences (Wrighton and Vandenbranden, Arch. Biochem. Biophys., 268, 144, 1989).

The regulation of the fetal cytochrome P-450 isozyme IIIA is not fully understood. Steroids e.g. estrogens or progestagens accumulating in the feto-placental unit may trigger the onset of synthesis of the enzyme. Therefore, the substrate specificity of the fetal enzyme is of crucial interest and currently investigated. Various aspects of the physiological regulation of the fetal enzyme will be discussed.

The rat has 3 isozymes of the cytochrome P-450 III family. Yet, this species has little value as an animal model since none of them is expressed prenatally. This is also true for the only III enzyme form known in the rabbit liver (Pineau et al, Abstr., VIII Int. Symp on Micr. & Drug Oxidation, 1990).

The human fetal liver also contains low (or trace) oxidation activities (probably) catalyzed by cytochrome P-450's of the I and II gene families. Cytochrome P-450 IID6, which is polymorphically expressed in humans, is however not present in the mid-trimester fetal liver as observed with a series of opioid substrates in our laboratory.

The regulation of fetal enzymes will be discussed in relation to the divergent ontogenetic enzyme development in man and experimental animals.

ENVIRONMENTAL FACTORS AFFECTING DRUG METABOLISM.

Olavi Pelkonen.

University of Oulu, Department of Pharmacology and Toxicology, SF-90220 Oulu, Finland

Variability in drug-metabolizing enzymes is determined by the interplay of hereditary, non-genetic host and environmental factors. Although the extent of exposure (dose) to an environmental factor is obviously very important - and in most cases also seemd to determine the magnitude of an outcome - specific final response after the exposure is always dependent on a "willing" host, i.e. the response is dependent on the host-environment interaction, in which an outside factor finds complementary host structure(s) and elicits a response. Without complementary structures, no specific interaction (and specific response) is possible. However, non-specific, secondary, consequences to drug metabolism can result from cell injury, necrosis, membrane perturbation and so on.

Numerous environmental factors affect drug metabolism both quantitatively and qualitatively. Exhaustive lists can be devised easily (because almost any exogenously induced condition seems to have an effect, if a suitable and sensitive measure is used), but it is more difficult to assess pharmacological or toxicological (or even more so clinical pharmacological) significance of any one factor. Some examples of specific responses of drug metabolism to environmental factors include P450-form-specific increases by cigarette smoking and/or polycyclic aromatic hydrocarbons, polychlorinated biphenyls etc in a wide variety of species, tissues and cells (P450IA1/2 induction via the Ah receptor interaction); by ethanol, acetone, pyrazole, isoniazid, hydrazine etc in the liver of several species including human (P450IIE1 induction); by pyrazole, cobalt, phenobarbital, TCPOBOP etc in mouse, and possibly human, liver (P450IIA3/4 increase); by macrolide antibiotics and certain glucocorticoids in rat liver (P450IIIA increase, probably not via the glucocorticoid receptor). However, in most cases detailed mechanisms remain obscure, although specific receptors in the classical pharmacological meaning of the word (except in the case of the Ah receptor) seem not to be involved. A typical non-specific response of drug metabolism is associated with the development of liver injury - and ensuing decreases in drug-metabolizing enzymes - caused for example by excess alcohol drinking or exposure to other types of toxic substances, although one must be aware that not all P450 forms or other drug metabolizing enzymes are equally susceptible to injurious factors. For example, glucuronyl transferase seems to be more resistant than oxidative enzymes to hepatotoxic agents.

Grant: The Academy of Finland Medical Research Council.

Expression and Regulation of Drug Conjugases

B Burchell, M W H Coughtrie, L Waddell, D Clarke, R Wooster, S Fournel-Gigleux*, Department of Biochemical Medicine, Ninewells Hospital and Medical School, University of Dundee, DD1 9SY, Scotland and *Centre du Medicament, Unite de Recherche Associee 597, Centre National de la Recherche Scientifique, Nancy France.

Conjugation with glucuronic acid, sulphate or water improves the water solubility of a large variety of endogenous and xenobiotic compounds, thereby facilitating their excretion into the bile or urine. Conjugate formation is mainly catalysed by a series of UDP-glucuronosyltransferases (UDPGTs), epoxide hydrolases (EHs), sulphotransferases and glutathione transferases (GSTs), protein products of multigene families. The functional heterogeneity of the rat and human transferases is gradually being resolved by protein purification and expression of cloned cDNAs.

Studies of the regulation of UDPGTs during perinatal development and by xenobiotics examined using antibody and cDNA probes will be described.

Human liver microsomal UDPGTs and EH have been expressed in cell cultures and yeast. Sulphotransferases are being cloned and expressed. Cotransfection and expression of these enzymes in various combinations will facilitate examination of the contribution of individual isoenzymes to conjugation of drugs catalysed by multiple enzymes. The identification of other sites of cellular regulation suggested from work with isolated hepatocytes can also be examined using these systems Regulation of transport throughout the liver cell will be discussed.

Activation of Oxygen in Metabolism and Toxicity
V. Ullrich
Faculty of Biology, University of Konstanz, Box 5560, D-7750 Konstanz

All higher forms of life require oxygen but this molecule is also very often or possibly always responsible for the termination of life. All reactions of oxygen require an activation step which invariably involves electron transfer reactions. Free electrons lead to superoxide, hydroperoxide or hydroxyl radicals, which are highly destructive when generated in biological systems. Activation of dioxygen for metabolic reactions proceeds via enzymic intermediates, which either contain metal ions or organic residues of flavins or unsaturated fatty acids. Dependent on the amount of transferred electrons and the degree of stabilization in the intermediate, a whole range of reactivities can be generated this way and used for the catalysis of oxygen transport, dioxygenations, monooxygenations or oxidase reactions. It is the purpose of this contribution to define the reactivities of all known species of activated oxygen and to describe their origin or enzymatic mechanisms of generation.

A second part will be devoted to the defense mechanisms of the cell against free oxygen species and the capacities of these systems to cope with increasing concentrations of such toxic intermediates. This will allow to define the conditions of "oxygen toxicity".

Abstracts not yet received:

M. Przybylski

APPLICATION OF IMMUNOLOGICAL METHODS IN DRUG METABOLISM

Felix Waechter

Central Toxicology Unit, CIBA-GEIGY Limited, CH-4002 Basel, Switzerland

As a defense against microbial infection, vertebrates produce immunoglobulins (antibodies) which contribute a major class of proteins found in blood. Antibodies are made in millions of different forms, each with the unique property to bind with high affinity to a limited region (epitope) of the molecule (antigen) which induced its production. This non-covalent antibody-antigen interaction involves mainly hydrophobic and ionic forces.

Repeated injection of an antigen sample (protein, peptide, carbohydrate, protein-bound hapten) into experimental animals induces the production of circulating antibodies by terminally differentiated B-lymphocytes. Antibodies obtained in this way are known as polyclonal antibodies. They have been widely used for a long time by cell biologists and biochemists as powerful tools. Labelled directly or indirectly (e.g. with enzymes, fluorescent dyes, radioactive isotopes or colloidal gold particles), they are invaluable for locating antigens on microscopical sections (at the light and electronmicroscopical level), for the detection and quantification of antigens (macromolecules as well as small organic molecules) in biological samples, for enzyme inhibition studies, for affinity purification and topological analysis of proteins, and last not least, in molecular biology for the isolation of specific cDNAs from cDNA-expression libraries.

The serum of an immunized animal contains a mixed population of antibodies against the antigen of interest. Each of these antibodies is secreted by an individual B-cell clone and recognizes a specific epitope. However, it is often desirable to work with an antibody preparation which specifically recognizes a particular epitope on the surface of an antigen (e.g. in order to characterize closely related proteins such as cytochrome P-450 isozymes). This can be achieved in different ways. Instead of producing antibodies against a whole protein, anti-peptide antibodies can be raised, which are able to recognize the amino acid sequence of this peptide also in the intact protein. Alternatively, an antibody secreting B-cell can be fused with a myeloma cell. The resulting hybrid cell line can be maintained in culture and will continue to produce one specific so-called monoclonal antibody.

Very recently, the production of an enormous diversity of antibody fragments in bacterial cells has been reported. This new ingenious antibody cloning technique could easily make the monoclonal antibody technique antiquated: The culturing of mammalian cells is completely bypassed and a nearly endless field for tinkening opened.

APPLICATION OF RECOMBINANT TECHNIQUES

J.Doehmer, F.Oesch, S.Dogra, C.Janssens, K.L.Platt, and H.R.Glatt

Institut für Toxikologie und Verfügungsbau f. Forschung u. Entwicklung Johannes Gutenberg-Universität Mainz, D-6500 Mainz, F.R.G.

Drugs as other xenobiotics are metabolized upon application. It is therefore of fundamental toxicological and pharmacological interest to identify the metabolites and to understand their effects on a molecular and cellular level. The classical procedure involves the use of animals for drug metabolism studies. However, due to the complexity caused by the potential involvement of so many different enzymes and their interaction it is extremely difficult to understand the conditions that lead to the generation of metabolites and to identify the enzymes involved.

As an attempt to escape this complexity cultivated cells were applied in drug metabolism studies. However, cultivated cells rapidly loose the capacity to metabolize drugs. The better a cell is adjusted to culture conditions the less highly differentiated metabolic functions are maintained. Especially the expression of key enzymes such as cytochrome P450s are lost.

Recombinant DNA technology has made it possible to overcome metabolic deficiencies in cultivated cells by DNA mediated gene transfer of cDNAs encoding cytochrome P450s and other metabolizing important enzymes. The general experimental strategy involves:

1. **Cloning** of full length cDNA from cDNA libraries;
2. **Recombination** with an eukaryotic expression vector;
3. **Gene transfer** into the recipient cell;
4. **Selection** and **characterization** of cell clones on DNA-, RNA-, and protein level;
5. **Enzymatic characterization;**
6. **Validation** of cell clones for drug metabolism;
7. **Application** in drug metabolism studies.

As a result cell lines are obtained that are highly defined for a particular function and which may serve as an analytical tool to understand drug metabolism on a molecular and cellular level.

Non-Invasive Methods in Drug Metabolism

J.H.M. Schellens, P.A. Soons & D.D. Breimer

Center for Bio-Pharmaceutical Sciences, Division of Pharmacology, University of Leiden, PO Box 9503, 2300 RA Leiden, The Netherlands.

Non-invasive methods in drug metabolism most often refer to the measurement of xenobiotics and/or their metabolites in urine, saliva and sometimes the feaces (bile). In humans determination of the excretion pattern of drugs and metabolites in the urine serves many purposes: 1 to obtain information on the biotransformation of a compound, 2 to use in exposure tests (biological monitoring), 3 to determine the phenotype of the acetylation and oxidation polymorphisms, 4 to use as a measure of the activity of the oxidative drug metabolizing enzyme system in order to establish interindividual differences and changes in drug metabolism. Biological monitoring has been accepted as a useful qualitative method to establish exposure levels of humans to toxic or mutagenic compounds as pesticides, arylamines and others. The acetylation polymorphism affects the elimination of clinically applied drugs as well as potential carcinogenic compounds. Therefore determination of the acetylation phenotype may be indicated. The urinary excretion of parent drug and acetylated metabolite(s) of probe drugs as isoniazide, dapsone, sulphadimidine and caffeine can be used conveniently to establish the acetylator status of a subject. Also the phenotype of the oxidative polymorphisms of sparteine/debrisoquine and mephenytoin can be established by determination of the ratio of parent drug and metabolite(s) excreted into the urine. Antipyrine oxidation and metabolite formation have been used extensively to assess the activity of the cytochrome P-450 system, the main oxidative drug metabolizing enzyme system. Clearance of antipyrine can be established accurately using a one-point measurement of the drug in saliva. The three main metabolites of antipyrine are excreted into the urine and they can be measured accurately by analyzing urinary samples collected up to approximately 5-times the expected elimination half-life. For the characterization of the oxidative drug metabolizing enzyme activity a 'cocktail' of probe drugs can be applied. There are at least two advantages associated with this approach, a practical one and a conceptual one. It is of great practical advantage if one is able to obtain information on the activity of different isozymes in one experimental session. For example phenotyping for different oxidative polymorphisms can be carried out in one experiment. The 'cocktail' approach is also of great advantage in metabolic correlation studies, with the aim to elucidate commonly regulated metabolic pathways, and in studies in which the influence of environmental factors, as enzyme induction and inhibiton, is to be assessed. Simultaneous administration of the probe drugs warrants that the metabolic oxidation of all substrates is studied under identical conditions. Thus intraindividual differences in drug oxidation with time are eliminated. In these types of experiments determination of the formation clearance of metabolites is essential, which implies analysis of the urinary excretion of metabolites. Conclusion: by non-invasive methodology relevant information can be obtained on the "metabolic status" of an individual subject, as well as on the changes in metabolizing enzyme activity. For quantitative purposes however often the evaluation of drug and/or metabolite kinetics in plasma is also indicated.

The use of computer-assisted molecular modeling in pharmaceutical research.

Klaus Gubernator

Hoffmann - La Roche AG, Central Research Unit, Basel.

Studies of drug-receptor interactions at a molecular level became possible in recent years through the availability of experimental structures of biopolymers at atomic resolution. These structures include proteins, protein-inhibitor complexes, DNA fragments, DNA-drug complexes, proteins bound to DNA, carbohydrates alone and in complexes with proteins. Major targets of todays pharmaceutical research are inhibitors of enzymes which belong to classes for which experimental data are now available. Detailed investigations of enzyme inhibition using these data requires powerful computer-methods due to the complexity of the systems considered: Databases of X-ray structures of proteins and small molecules together with efficient retrieval software; real-time three dimensional display systems; efficient force-field methods and fast computers; tools for visual analysis of interactions; manipulation and modification tools for molecules of any kind.

Three proteins who's structure has been determined at Roche will be used to illustrate the application of our systems to recent inhibitor-projects: Thrombin[1], a serine protease of the blood coagulation cascade, a class C betalactamase[2] and human pancreatic lipase[3]. The mechanism of substrate cleavage and the mechanism of inhibition with drug-size molecules will be discussed. A common stereo-electronic machinery shared by the active site of all three enzymes has been discovered and could have some impact on future research.

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MECHANISMS OF CYTOTOXICITY

Peter Moldéus

Department of Toxicology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden.

Despite intense research the mechanism whereby chemicals kill cells are still not known. Several events have been suggested to be pivotal for the development of cytotoxicity induced by chemicals and other noxious stimuli. These events include lipid peroxidation, covalent binding to tissue proteins, protein thiol oxidation, depletion of pyridine nucleotides and ATP, DNA damage and disturbance of Ca^{2+} homeostasis. One or several events may then lead to toxic cell damage where the cell death is due to an altered cellular biochemistry and failure of the endogenous systems to compensate.

Eventhough different chemicals induce different toxic events there is evidence that there may be a common pathway by which cytotoxicity is induced in different cell types by different agents. One such common pathway could be the perturbation of Ca^{2+} homeostasis which in turn may be due to modifications of SH-groups in key enzymes.

Intracellular Ca^{2+} homeostasis is normally maintained by the concerted operation of cellular transport and compartmentation systems. However, impairment of these processes during cell injury can result in enhanced Ca^{2+} influx, release of Ca^{2+} from intracellular stores, and inhibition of Ca^{2+} extrusion at the plasma membrane. This can lead to uncontrolled, systained rises in cytosolic Ca^{2+} concentration, which differ from the rapid, physiological transients observed in response to hormones, and are invariably associated with cell death. Although cytotoxicity can occur by mechanisms that are independent of Ca^{2+}, several other processes have recently been identified by which sustained increases in intracellular Ca^{2+} level can activate cytotoxic mechanisms. These include the disruption of the cytoskeletal network and an uncontrolled activation of Ca^{2+}-stimulated catabolic enzymes such as phospholipases, proteases and endonucleases.

In the presentation the potential roles of the various processes in cytotoxicity will be discussed.

METABOLISMUS IN VITRO / IN VIVO

K.L. Platt, K. Marx, E. Molitor, H. Maith, H. Ruß*, D. Utesch, B. Diener, K. Mendla*, V. Häselbarth*, W. Tröger*, W. Pollmann* and F. Oesch

Institute of Toxicology, University of Mainz, D-6500 Mainz, FRG and * Department of Biochemistry, Boehringer Ingelheim KG, D-6507 Ingelheim, FRG

Lipophilic xenobiotics have to be metabolized by cellular phase I and phase II enzymes in order to be biliarily and renally excreted. In many instances metabolism, especially by phase I enzymes, may lead to cytotoxic and/or genotoxic metabolites. In the case of drugs, intended for human use, the latter situation has to be recognized in the early stage of drug development. For this purpose chronic toxicity testing has to be performed with two animal species, which exhibit a similar metabolic specificity as man. It is not certain which animal species fulfills this requirement, nor will it be the same species for each drug. Thus in many cases this expensive proceeding wastes a great number of experimental animals without leading to results relevant for the human situation.

We, therefore, propose to develop a battery of in vitro metabolizing systems derived from different experimental animal species and from man that can be used to find at an early time point (before in vivo metabolism in man is allowed) the suitable animal species for chronic toxicity testing which most closely resembles the metabolic specificity of man towards a given drug. Since the liver plays the predominant role in the biotransformation of most drugs freshly isolated hepatocytes represent a suitable in vitro model for drug metabolism studies. In cases, however, were fresh liver is only rarely available (large experimental animals, man) cryopreservation of hepatocytes can provide in vitro systems enzymatically stable at -196 °C for months. In order to check the suitability of our in vitro/in vivo metabolism concept the biotransformation of the following drugs in the rat in vivo and in vitro was determined: clonidine, alinidine (2-[N-allyl-N(2,6-dichlorophenyl)amino-2-imidazoline) and apafant (4-(2-chlorophenyl)-9-methyl-2-[3-(4-morpholinyl)-3-propane-2-yl]-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine). This comparison revealed a good qualitative and a satisfactory quantitative correlation between the metabolic situation in the rat in vivo and in vitro.

This work is now extended to a further drug (Web 1881, 4-aminomethyl-1-benzylpyrrolidin-2-one) and to other animal species (mouse, rabbit, dog, pig, monkey) and man. The results should prove whether in vitro metabolism studies can be used to select an animal species for chronic toxicity testing mimicking the metabolic capacity of human liver in vivo.

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METABOLIC AND DISPOSITIONAL BASIS OF TARGET ORGAN TOXICITY

John Caldwell

Department of Pharmacology and Toxicology, St. Mary's Hospital Medical School, London W2 1PG, England.

Xenobiotics are not uniformly hazardous to the cells of the body but rather exert their effects selectively and the tissues liable to damage by a particular compound are referred to as the target organs of toxicity. Three origins may be advanced for the particular liability of specific target organs to chemically-induced damage (i) the bioaccumulation of a chemical within the target organ, (ii) the presence of a target mechanism for toxicity within the target organ and (iii) metabolic activation of a toxic chemical within the target organ. Examples of toxic chemicals which are concentrated within the target organ include paraquat (lung), streptotozocin (β-cells of pancreas), cephalosporins (kidney) and compounds whose binding to melanin underlies their retinotoxicity or ototoxicity. There are relative few compounds whose organ-specific toxicity depends upon biochemical and/or physiological peculiarities of the target organ, but this is seen with those organophosphorus compounds which are substrates for the "neurotoxic esterase" of the CNS. The role of metabolic activation in target organ toxicity is rendered problematical by two contradictions (i) although the liver is the major site of metabolism, it is remarkably resistant to chemical injury and (ii) metabolically produced reactive intermediates are generally "short range toxins", too reactive to reach other tissues via the systemic circulation. Four types of metabolic activation underlying target organ toxicity may be discerned. Clearly, reactive intermediates may be produced within the target tissue itself as is seen with carbon tetrachloride (liver) and 4-ipomeanol (Clara cell of the lung). Different metabolic pathways may operate in different target organs : the nephtotoxicity of phenacetin is due to free radical intermediates produced after N-deacetylation while its hepatotoxicity is mediated by its O-deethylation product paracetamol. Second, some reactive intermediates formed in the liver are in fact stable enough to exert effects in remote organs *e.g.* N-oxides of pyrrolidizine alkaloids and bromobenzene oxide, both formed in the liver and toxic to the lung. Third, metabolic activation is often a multi step process and these steps may occur in different organs. Thus, the nephrotoxicity of various haloalkanes is due to the metabolic activation in the kidney of glutathione conjugates formed in the liver. Lastly, target organs may be able to reverse detoxication pathways, liberating a toxic compound at its site of action : this underlies the bladder toxicity of allyl isothiocyanate and 2-naphthylamine, where conjugation with glutathione and glucuronic acid, respectively, serves to produce a transport form of the toxin rather than a readily eliminated detoxication product.

INTERACTIONS BETWEEN ENDOGENOUS AND XENOBIOTIC METABOLISM AND TOXICITY

Josef Gut

Department of Pharmacology, Biocenter of the University, Klingelbergstrasse 70, CH-4056 Basel

Most xenobiotics (to which man is continually exposed therapeutically, occupationally, behaviorally or environmentally) require metabolic activation before tissue toxicity becomes evident. Adverse toxicity reactions can be classified on the basis of their mechanism of producing damage into intrinsic and idiosyncratic toxicities. Intrinsic toxicities of compounds may predictably occur based on chemical properties of the compound, on dosage, or on peculiar metabolism of compounds by distinct individuals (if, for example, being afflicted with a polymorphism in drug oxidation). In contrast, idiosyncratic toxicities are not a property of the compound, are not predictable, are host and dose independent, and are thought to have an immunological basis.

On the molecular level, the initial interaction between endogenous metabolism with that of xenobiotics may cause an intermingling of basic features, which eventually lead to the expression of one or the other type of toxicity. Parent compounds or metabolites thereof may interfere in intra- or intercellular signaling pathways, may inactivate through covalent modification components critical in cell functioning, and may activate/deactivate distinct tissue resident or peripheral cell types; such events may i) evolve into direct cytotoxicity; ii) give rise to covalently modified cellular macromolecules that may function, in susceptible individuals, as neo-immunogens in the development of idiosyncratic reactions, or iii) lead to a derangement of the endogenous homeostasis of auto- and paracrine factors.

There is ample evidence for the complexicity of such interactions. Upon being metabolized, hepatotoxins (acetaminophen, halothane, possibly others) induce, through as of yet not fully understood mechanisms, the formation of chemotactic factors in hepatocytes, which in turn cause an infiltration into the liver of circulating macrophages. Furthermore, liver resident macrophages (Kupffer cells) are carrying polypeptides covalently modified through adduct formation with reactive drug metabolites. Functionally activated immigrant and liver resident macrophages are active in both phagocytosis and release of superoxide anions; they are also the likely source for the local secretion of prostaglandins, leukotrienes, and monokines (among them interleukin-1 and tumor necrosis factor-α). Considerable efforts will be necessary to more profoundly understand the role of an inbalance of such endogenous mediators in the etiology of toxic syndromes in the presence of xenobiotics or metabolites thereof.

IMMUNOLOGICAL AND METABOLIC BASIS OF DRUG HYPER-SENSITIVITIES

J. Gerry Kenna

Department of Pharmacology and Toxicology, St. Mary's Hospital Medical School, Norfolk Place, London W2 1PG, UK.

Much of our current understanding of the mechanisms by which drugs and environmental chemicals may cause immune mediated adverse reactions ("hypersensitivities") is dominated by the "hapten hypothesis". This states that the drug, which usually is much too small to elicit an immune response directly, must first bind to a large carrier macromolecule, either by a very strong non-covalent interaction or by a covalent reaction. For drugs which are unreactive, metabolic bioactivation to reactive metabolite(s) will be required. Subsequently, the drug-macromolecule conjugate (or drug metabolite-macromolecule conjugate) becomes immunogenic in susceptible individuals, and elicits an immune response which causes the observed toxicity. The "hapten hypothesis" is supported by various *in vitro* studies. Covalent binding of drugs which elicit hypersensitivity reactions to plasma and tissue macromolecules has been demonstrated, as has bioactivation and covalent binding of unreactive drugs. In patients with drug hypersensitivities, humoral and cellular sensitisation to drug-like haptens (or drug metabolite-like haptens) bound to various defined carrier macromolecules has been reported. Relatively recently, autoantibodies which recognise the hepatic cytochrome P-450 isozyme responsible for metabolism and bioactivation of tienilic acid have been demonstrated in patients with tienilic acid-induced hepatitis. Furthermore, antibodies that recognise novel antigens, consisting of a metabolite of halothane bound covalently to several liver microsomal proteins, have been shown in patients with halothane-induced hepatitis. In this latter example, the epitopes recognised by patients' antibodies appear to consist of the bound halothane metabolite in conjunction with unique structural features of the various protein carriers.

From the "hapten hypothesis" it follows that the relative rarity and unpredictability of drug hypersensitivity reactions might be be attributable to intra-population variability in drug metabolism and/or immune responsiveness. Evidence that both factors can be of importance is available. Thus, acetylator phenotype has been implicated in susceptibility to procainamide or hydralazine-induced lupus erythematosus, while both sulphoxidation status and MHC phenotype have been implicated as risk factors in adverse reactions to penicillamine.

Further progress in this field will be dependent upon more detailed delineation of pathogenic mechanisms. This will in turn require detailed characterisation of the macromolecular target antigens against which patients' immune responses are directed, which is now technically feasible, as illustrated by the progress being made on tienilic acid-induced hepatitis and halothane-induced hepatitis.

TOXICOKINETICS AND METABOLISM IN RISK ASSESSMENT.

Trevor Green

ICI Central Toxicology Laboratory, Alderley Edge, Macclesfield, Cheshire, SK10 4TJ, UK.

Information about the adverse properties of chemicals is usually obtained from experiments using laboratory animals, often exposed to dose levels significantly higher than those normally experienced by humans. Consequently, in the determination of human risk from such studies, it is necessary to extrapolate both between dose levels and between species. Current methods rely on no effect levels and safety margins, or on linear dose extrapolation and some form of allometric scaling between species. Both methods are unable to take into account some of the more marked species, sex or strain differences in response that are all too frequently seen in laboratory animal experiments. Many chemicals are activated by metabolism to their toxic or carcinogenic forms and it is within the processes of adsorption, distribution, metabolism and excretion (ADME) that many of the species differences in response have their origins. Since ADME data is relatively easy to acquire and can have such a large impact on the biological response, it follows that such data should be incorporated into the risk assessment process. Models are now available to use this type of information, together with species-specific physiology, in the calculation of risk. The experimental animal and human data needed for these models often requires the use of a combination of in vivo and in vitro techniques. For example, human data is often only accessible from in vitro experiments using the relevant human tissues. Examples of how this data is acquired and of how it can be used to predict the uptake and metabolism of chemicals in humans in vivo will be given. These predictions then form the basis of the risk assessment.

Biotechnological Products as Drugs - Metabolism Hazards and Future

Prof Dr med Jürgen Drews
F Hoffmann-La Roche Ltd, CH - 4002 Basel

Proteins have played a modest but distinct role as drugs for many decades. Insulin, human growth hormone and coagulation factors, to name but the most important ones, have been in use for many decades. Biotechnology, and in particular gene technology, have recently moved proteins to the centre stage of drug research. While the influence of molecular biology on drug research is not confined to the provision of proteins with therapeutic potential, we must be aware that eight (8) new recombinant proteins have already been registered and that between 50 and 100 are in some stage of clinical development. Even if only a third of these entities will reach the market (a pessimistic estimate) at least 30 new protein drugs would have to be expected to enter the medical arena over the next few years. Moreover, new proteins which are presently still in the experimental stage will add to this figure in the more distant future.

In principle, proteins like various cytokines, enzymes, adhesion proteins and fusion proteins which comprise receptor binding ligands fused to toxin or antibody moieties must be evaluated according to well established criteria of drug therapy: they must be safe, effective and they must be produced in controlled ways which ensure the physical identity, purity and stability of the eventual product.

Since cytokines, proteins which induce growth, differentiation and the activation of cells, form the major proportion of the new class of recombinant therapeutics, the issue of efficacy (experimental and clinical) as well as of kinetic parameters related to efficacy will be discussed in the context of these substances. Secondly, special aspects relating to the physico-chemical identity of protein drugs, their purity and their safety will be discussed.

Finally, new developments relating to the use of proteins and in particular cytokines as drugs will be presented. While the application of modern drug delivery systems to interferons or interleukins may seem feasible, the ideal solution could - at least in some cases - reside in the local release and application of cytokines. This can possibly be achieved by engineering cytokine genes into human cells, e.g. lymphocytes, which can be grown in vitro and reinfused into their donors. These cells could then be expected to migrate into tumours or other foci of disease and release their specific cytokines in response to a chemical signal which would have to be applied separately.

The use of recombinant proteins as drugs is likely to have a major impact on the therapy of certain diseases, especially malignancies, allergic conditions, chronic inflammatory diseases, infections and traumatic lesions. Therefore, it seems mandatory for pharmaceutical R & D organizations in major drug houses, to familiarize themselves with some special features of proteins which must be addressed before any of these substances can become drugs.

Abstracts not yet received:

P. Dayer

Stereochemical aspects of drug Metabolism

M. Eichelbaum

Dr. Margarete Fischer-Bosch-Institute für Klinische Pharmakologie, Auerbachstr. 112, D-7000 Stuttgart 50/FRG

Whereas differences in activity between stereoisomers have been known for many years, only recently has the importance of stereoselectivity in drug disposition been realized. The process of drug metabolism involves an interaction between the drug molecule and chiral biological macromolecules. Therefore, it is not surprising that in analogy to the differences in pharmacological activity, the interaction of stereoisomers with the drug metabolizing enzymes will exhibit a certain degree of handedness. As a consequence stereoselective drug disposition occurs. It is often overlooked that the stereoselectivity in the activity of enantiomers as determined in vitro cannot be extrapolated to the in vivo situation since stereoselective drug metabolism can lead to an enantiomer ratio in vivo which differs substantially from that in the dosage form administered. Stereoselectivity in drug metabolism seems to be the rule rather than the exception and, depending on whether the active or less active enantiomer is preferentially affected there maybe amplification or attenuation of in vivo as compared to in vitro drug potency. Numerous pathways of drug metabolism display stereoselectivity. In this regard, a distinction must be made between substrate and product stereoselectivity. In the case of substrate stereoselectivity enantiomeric substrates may be metabolized at different rates and/or by different routes, leading to preferential metabolism of one enantiomer. (The rate of metabolism of verapamil by O-demethylation is at least ten times greater for S-than for R-verapamil. 7-hydroxy warfarin is almost exclusively formed from S-warfarin).

If active metabolites are formed from a racemate where the enantiomers of the parent drug differ in potency the contribution of the active metabolite to the overall effect very much depends whether the eutomer or distomer is preferentially transformed into this metabolite. Metabolism can lead to loss of chirality (chiral-achiral transformation) as it has been shown for chiral dihydropyridines where oxidation to pyridine transforms the chiral carbon atom at position 4 of the dihydropyridine ring into an achiral atom.

By contrast product stereoselectivity, which implies the differential formation of stereoisomeric metabolites from a single substrate containing one or several prochiral centers, is quite often characterized by a high degree of stereoselectivity leading to the almost exclusive formation of only one of two possible enantiomeric metabolites. This is in all probability due to the metabolite being formed by only one specific enzyme. For example the antihypertensive drug debrisoquine is achiral, however, the carbon atoms in positions 1,3 and 4 are prochiral. The major metabolite results from hydroxylation at prochiral atom 4, thereby introducing a chiral center. Thus, 4-hydroxy-debrisoquine could be formed as either the R- or the S-enantiomer. In extensive metabolizers almost exclusively the metabolite with S-configuration is formed.

A unique and interesting pathway of stereoselective metabolism has been established for 2-arylpropionic acid derivatives. Via formation of an acyl coenzyme-A-thioester of the inactive R-enantiomer, and racemization of this complex by a Co A racemase a chiral inversion to the active S-enantiomer occurs. Stereoselective drug metabolism may have important consequences in the case of drug interactions if differences in potency exist between enantiomers and the disposition of the enantiomers is variously affected by the causative drug. For example a drug may mainly inhibit the metabolism of the eutomer and may induce or not affect the metabolism of the distomer. Therfore, despite an unchanged or even decreased total plasma concentration, an increase in activity can be observed. On the other hand, if mainly the distomer and not the eutomer is affected, the higher total concentration which results is of no or only minor consequence for the therapeutic effect.

POLYMORPHISMS OF DRUG METABOLISM AND HOW TO DETECT THEM

Denis M. Grant
Division of Clinical Pharmacology and Toxicology, Hospital for Sick Children, Toronto, Canada M5G 1X8

The goals of pharmacogenetic investigations are the discovery, detection, mechanistic elucidation, accurate prediction and ultimately the prevention of genetically determined clinical manifestations of variable drug response in human populations. In particular, genetic polymorphisms of drug biotransformation capacity have been the focus of much study in recent years because of their well-documented consequences for clinical therapy, drug development and toxicology. Such polymorphisms share three common features relevant to their detection: 1) they are monogenic -- observed variations arise from differences in expression of alleles at a single gene locus; 2) by definition, they result in the occurrence of a distinguishable variant phenotype which is not rare; and 3) the variant phenotype is normally silent in the absence of drug challenge.

In general, the detection of pharmacogenetic variants can be approached from two different angles. The first and more classical approach is event-based. Since the defective phenotype is expressed only after exposure to a drug or chemical, this expression often takes the form of drug-induced toxicity. Once this has occurred, retrospective studies can then be used to ascertain genetic involvement in producing such toxic events. Experimental protocols for doing this include the study of family pedigrees arising from an affected propositus to test for Mendelian inheritance of the trait, and comparison of monozygotic vs. dizygotic twins to arrive at heritability estimates for the trait. The second approach, which our current awareness of the potential for interindividual variation should make more popular for future drug development, is drug-based. Starting with a particular chemical structure, animal and *in vitro* studies with human tissues can help to predict possible sites for pharmacogenetic variability. Then, a prospective, detailed examination of the type of variation associated with a drug's administration to relatively large groups of subjects can give indications as to whether genetic factors can indeed be expected to play a significant role in its disposition. A more directed form of this approach is the use of a "typed-panel" comparison, where the disposition of a drug is evaluated in a subgroup of the population selected for their known metabolizer phenotype, to verify suspected involvement of of a specific variant enzyme in producing observed variability.

Obviously such screening procedures are themselves associated with a risk of producing toxicity if genetically predisposed individuals exist in the tested population sample. For this reason *in vivo, in vitro* and molecular genetic tests for population and patient screening which minimize or avoid toxic drug exposures continue to be developed for use in pharmacogenetic studies. Still the most popular of these is the administration of relatively non-toxic "probe" drugs (i.e. caffeine for acetylator phenotyping) whose pharmacokinetics or urinary metabolite profiles can be used to establish metabolizer phenotypes in population samples. It is also possible to directly determine variations in the activity of certain drug-metabolizing enzymes in accessible blood cells *in vitro* once it has been established that the pattern of expression of the activity in these cells is representative of that in the major organs of xenobiotic biotransformation. Most recent, however, is the advent of genotyping procedures using recombinant DNA methodologies which can be used to screen large populations without exposure to potentially toxic drugs. Such methods, which include restriction fragment length polymorphism (RFLP) analysis by Southern blotting, allele-specific oligonucleotide hybridization, and related techniques using enzymatically (PCR-) amplified DNA segments, have been successfully applied to the study of genetic polymorphisms of debrisoquine 4-hydroxylase (cytochrome P450IID6), arylamine N-acetyltransferase (NAT2) and glutathione transferase.

Drug Metabolism in Disease States

Johannes Bircher, University of Witten/Herdecke, FRG

When drugs are metabolized in an organism the rate of metabolism and the pattern of metabolites depend not only on the enzymes available for this purpose, but also on the physiological processes which govern the interaction between substrates and enzymes. In disease states either the enzymes, or the cells harboring the enzymes or the physiological processes which carry substrates or cosubstrates to the enzymes may be altered (1,2).

Alterations of the enzymes occur mainly as a result of drug interactions or of toxins. This may be particularly important for drugs which normally are given by mouth and undergo a significant first-pass elimination. In these instances inhibition e.g. by ethanol and enzyme interaction e.g. by antiepileptic drugs may dramatically increase or decrease the systemic availability of drugs. Alternatively, drugs with relatively long half-lives which are used for long-term treatment may achive correspondingly low or high steady state concentrations. The number of hepatocytes available for drug metabolism may be mildly to drastically reduced in acute or chronic hepatitis and in cirrhosis. This affects the same processes which are altered by inhibitors of drug metabolism.

Altered physiological processes which can influence drug metabolism are mostly related to increases or decreases in hepatic blood flow and to portal systemic shunting. This affects particularly drugs with a high hepatic extraction, because their clearance is flow limited. When given by mouth the normally occurring first-pass elimination may be bypassed and the resulting increase in systemic availability may lead to toxic concentrations. When studying such physiological changes sorbitol may be an ideal reference compound (3,4).

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ASSESSMENT OF ENZYME INDUCTION AND ENZYME INHIBITION IN MAN

B.K. Park

Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX.

The duration and intensity of pharmacological action and the toxicity of many xenobiotics are dependent, in part, on their rate of metabolism. With regard to toxic metabolite formation, the balance between bioactivation and detoxication will be a critical determinant of xenobiotic toxicity. Recognition of these important roles for drug metabolism has stimulated a great deal of research into factors which induce or inhibit the activity of the enzymes responsible for the various types of biotransformation that occur in man. Individuals vary in their ability and capacity to metabolise drugs. For the safety assessment of drugs and other chemicals to which man is exposed, there is a need to develop methods with which to identify those chemical compounds which are potential enzyme inducers and enzyme inhibitors in man, and also to assess the response of an individual to such compounds. The principal methods that have been used for this purpose are 1) determination of the pharmacokinetics of a model compound by analysis of blood, saliva, expired air or urine 2) analysis of drug metabolism *in vitro*, at one time a very limited process, but now more readily available with the development of human liver banks, which have proved especially useful for the investigation of enzyme inhibition, 3) observation of changes in the concentrations of endogenous compounds such as cortisol and oestradiol which are substrates for cytochrome P450 IIIA enzymes which are also responsible for the metabolism of a number of drugs such as cyclosporin and nifedipine. Cytochrome P450 enzymes from the liver and other tissues are directly involved in a number of drug-induced toxicities and drug interactions. A number of probe drugs for oxidation are available which can be used safely in man and also in parallel *in vitro* studies. Recent progress in the characterisation of the cytochrome P450 gene super family and the selectivity of individual forms of the enzymes, with respect to both substrates and inhibitors, has greatly improved the interpretation of data obtained with model compounds. However, extrapolation from *in vitro* experiments to the clinical situation requires a careful consideration of the concentration of the substrates in the two situations which will influence both enzyme-substrate selectivity and substrate turnover. Combined pharmacokinetic/pharmacodynamic studies are required to assess the pharmacological relevance of either induction or inhibition. However in order to assess the toxicological implications of enzyme induction and enzyme inhibition, it has been necessary to develop *in vitro* systems which comprise an activating system (eg. human liver microsomes) and human target cells (eg. lymphocytes/hepatocytes). Using such systems it is possible to investigate the balance between drug activation (eg oxidation) and drug detoxication (eg epoxide hydrolysis; glutathione transferase) and how this balance might be perturbed by other drugs.

DRUG METABOLISM IN CLINICAL TRIALS. WHAT DATA ARE NEEDED ?

Luc P. Balant[1,2] and Androniki E. Balant-Gorgia[1]

Department of Psychiatry[1] & School of Pharmacy[2], University of Geneva, P.O. Box 79, CH-1211 Geneva 8, Switzerland.

Ideally, the registration file of a new drug should contain the pharmacokinetic studies necessary to substantiate the choice of dosage regimen, and provide the useful information related to the security of the drug in the target population. Clearly, if all the necessary information has been obtained during the "formal" pharmacokinetic program, no further metabolic data need to be gathered from the patients participating to phase II or III clinical trials. This is, however, seldom the case. It is thus important to use all available information from clinical studies in order to explore the possibilities that drug disposition might be altered in particular patient sub-populations.

There are different strategies that can be used for this purpose. The simplest approach is a single point measurement of the steady-state concentration of the parent compound (i.e. the simple pharmacokinetic screen) this allows the detection of patients with abnormally high concentrations, but provides no information concerning the reasons of this abnormality. Furthermore, no information can be obtained for very efficient metabolizers, since they are mixed up with non-compliant patients. A second strategy is the population kinetic approach. It is then possible to calculate the systemic availability, the volume of distribution and the clearance of the drug, and to detect the co-variables (age, disease, co-medication ...) susceptible to modify these parameters. The main limitation of this approach is the need of more than one measurement per patient with a precise knowledge of drug dosing and blood sampling. Here again, non compliance represents a major bias. A third possibility is given if parent drug and metabolites (active or not) are measured on a single occasion at steady-state.

During this presentation the advantages and inconvenient of this latter approach will be discussed with data obtained from 150 patients treated with Anafranil. A single blood sample was taken for each patient at steady-state. Clomipramine, desmethylclomipramine, hydroxy-clomipramine and hydroxydesmethylclomipramine were measured in whole blood by HPLC. A clearance-based model allowed the construction of a nomogram from which the hydroxylation and demethylation clearances could be estimated in individual patients and in patient sub-populations. The influence of age, smoking, chronic alcohol intake, and phenothiazine co-medication on the two metabolic pathways were evidenced using simple statistical tests.

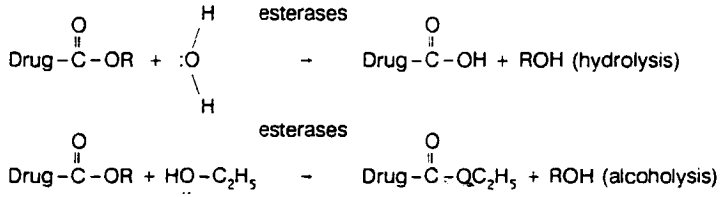
POSTERS I (P 1.1 – 1.44): REACTIONS AND ENZYMES

ENZYMATIC ALCOHOLYSIS, A RISK OF ARTEFACT FORMATION IN DRUG METABOLISM STUDIES.

Andersson Paul and Lindberg Claes.

AB DRACO Research and Development Department, P.O. Box 34, S-221 00 Lund, Sweden

Ethanol and other alcohols are effective nucleophiles towards acyl-groups in esterase catalysed reactions. In drug metabolism studies in vitro where alcohols often may be used as solvents for lipophilic drugs, alcoholysis instead of hydrolysis may occur, resulting in an enzymatic transesterification of acyl-containing drugs.



During incubation of some glucocorticoid carboxylic acid esters with human lung homogenate in the presence of low concentrations of ethanol or methanol (0.1%), we discovered that these compounds underwent enzymatic alcoholysis besides enzymatic hydrolysis. The transesterified derivatives resulting from alcoholysis were structurally identified by liquid chromatography and chemical ionization mass spectrometry.

The results from the incubations showed that increasing alcohol concentrations caused an increase in alcoholysis at the expense of hydrolysis and in some cases an altered total reaction rate (hydrolysis+alcoholysis) resulted.

The data stress the importance of avoiding the presence of alcohols in drug metabolism studies of acyl-ester drugs. Another aspect of alcoholysis may be of clinical relevance i.e. altered kinetics of these compounds during ethanol intake.

PURIFICATION AND CHARACTERIZATION OF A CYTOCHROME P-450 CORRESPONDING RAT P-450 IIB FAMILY IN THE MOUSE BRAIN MICROSOMAL FRACTION.

Z. AMELIZAD[1], J. F., NARBONNE[2], B., VOLK[3], R., KNOTH[3], J. C., GARCIN[2] and F., OESCH[1]

1-Institut für Toxicologie - University of Mainz - 6500 MAINZ - F.R.G.
2-University of Bordeaux I - Department of Food Toxicology - F.
3-Department of Neuropathology, - University of Frieburg - F.R.G.

Abstract:

Cytochrome P-450 has been found throughout the brain microsomal fraction of control mouse and phenytoin treated animals at a level which ranges between 30-400 pmol/g of tissue (AMELIZAD *et al.*, 1990), which is able to present immunological reactivity and catalytic activities (VOLK *et al.*, 1988). In this study, for the reason of physiological functions of brain P-450 and the possible involvement of these enzymes in toxicology, we tried to purifie these enzymes from total brain microsomes by using an affinity chromatography omega-diaminooctyl sepharose - 4B and DEAE ion exchange chromatography. The major purified enzyme presents a single band in S.D.S. gel electrophoresis and reacts immunologically with antibodies to rat cytochrome P-450 IIB1 and IIB2 (NEBERT *et al.*, 1987). This enzyme presents cataclytic activities for several substrates at a low level compared to rat P-450 enzyme but detectable activity in reconstituted systems.

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The P-450 gene superfamilly, recommended nomenclature
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Cytochrome P-450 multifamilly from cerebral fractions
Manuscript submitted

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First evidence of cytochrome P-450 induction in the mouse brain by phenytoin
1988, Neuroscience Letter 84 : 219-224

GENERATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO RAT LIVER 4-OH-BIPHENYL-UDP-GLUCURONOSYLTRANSFERASE

C.Augustin[1], F.Donn[2], A.Schmoldt[1],
Inst.for Legal Medicine, University of Hamburg, Butenfeld 34, D-2000 Hamburg 54[1]
Marienkrankenhaus, D-2000 Hamburg 70[2]

As reported recently (Naunyn-Schmiedeberg's Arch.Pharmacol., Vol.337 Suppl.,R14) a UDP-glucuronosyltransferase (UDP-GT) could be purified that glucuronidates 4-OH-biphenyl and methylumbelliferone but does not show activity towards morphine, testosterone and androsterone.

Two monoclonal antibodies could be developed against this UDP-GT-isoenzym, purified from liver microsomes of phenobarbital pretreated Wistar rats. Female Balb/c mice were immunized four times with 10 µg enzyme protein per week and complete or incomplete Freund adjuvans. Fusion of spleen and myeloma cells (P3X63-Ag8.U1) was performed with conventional techniques and hybridomas were grown and screened by means of the dot-blot technique with rat liver microsomes as antigen. Positive clones were further grown and retested on western blots.

MAbs BI8-2 and BI7-2 showed slightly different staining characteristics on western blots with an affinity purified alkaline phosphatase conjugated goat-anti-mouse IgG-IgM antibody.

Both stained one band in the 58 KD region using purified 4-OH-biphenyl-GT as antigen and did not stain any band using purified 4-Nitrophenol-GT, 17β-OH-steroid-GT, 3α-OH-steroid-GT or a fraction containig activity towards both morphine and 4-OH-biphenyl. MAb BI8-2 and MAb BI7-2 differed when liver microsomes were used as antigen. Whereas MAb BI8-2 stained one 58KD band MAb BI7-2 stained two bands, one in the 58 KD and one in the 56 KD region. The same pattern was obtained with microsomes of 5,6-benzoflavone pretreated or control rats, but the bands were less intense.

EVALUATION OF THE CONTRIBUTION OF THE P-450 III GENE FAMILY TO THE METABOLISM OF THE ERGOT ALKALOID CQA 206-291 IN RAT AND MAN.

S.E. Ball, U.A. Meyer*, G. Maurer and A. Vickers.

Biopharmaceutical Dept., Sandoz Pharma and *Department of Pharmacology, Biocenter of the University, Basel, Switzerland.

The ergot alkaloid CQA 206-291 is a novel dopamine agonist intended for the treatment of Parkinsons disease. We have shown using chemical inhibition studies that CQA metabolism is inhibited by testosterone, estradiol and cyclosporine suggesting an important role for the P-450 III gene family in the metabolism of this compound. In order to establish the contribution of this gene family to the metabolism of CQA 206-291, immunoinhibition studies were performed with rat and human liver microsomes using a polyclonal rabbit anti-rat PCN-1 antibody.

Microsomal incubations contained CQA (10µM; 1µCi), microsomal protein (5µg human, 7.5 µg rat), anti or preimmune serum (1µL/µg protein) and NADPH (1mM) in 0.1M potassium phosphate buffer(100µL). Ether extracts of microsomal incubations were analysed by radiometric hplc.

Human liver microsomal incubations yielded a single major metabolite peak, the formation of which was completely inhibited by the antibody. However the formation of two minor metabolites of CQA was not affected. Titration of the antibody showed that inhibition was concentration dependent and was complete at 0.2µL/µg protein. These results suggest that the cytochrome P-450 III gene family plays an almost exclusive role in the metabolism of CQA in man. Minor pathways of metabolism appear however to be catalysed by other gene families which remain to be elucidated.

Rat microsomal incubations produced two major metabolite peaks. One of these peaks was not inhibited by the antibody. The other peak which had an identical retention time to the major human metabolite could be inhibited by 50% indicating only a partial involvement of the III gene family in the rat.

In conclusion, there is an important species difference regarding the contribution of the P-450 III gene family to the metabolism of CQA in rat and in man. Any compound which will induce or inhibit this gene family could have an important effect on the disposition of this compound in man.

INTER-SPECIES VARIATIONS IN CAFFEINE METABOLISM RELATED TO CYTOCHROME P-450IA IN MAMMALS

F. Berthou[1], B. Guillois[1], P. Beaune[2], E. Jacqz-Aigrain[3], A. Guillouzo[4] and C. Riché[1]

1: Laboratoires de biochimie et pharmacologie Faculté de médecine-BP-815-29285-Brest (France); 2: INSERM U-75, Hôpital Necker, Paris; 3: Laboratoire de pharmacologie, Hôpital Robert Debré, Paris (France); 4: Unité INSERM U-49, Hôpital Pontchaillou-35033 Rennes (France)

Caffeine (1,3,7-trimethylxanthine) has been proposed as a chemical probe for monitoring the hepatic cytochrome P-450IA (PAH-inducible) in humans. This cytochrome P-450 (P-450) family contains two enzymes, namely IA1 and IA2 which differ by their catalytic activities, regulation and expression in mammals. Until now, it is not clear which enzyme(s) is (are) involved in the primary steps of caffeine metabolism.

This study has been carried out in order to understand the inter-species (man, monkey, rabbit, rat, mouse) differences in caffeine metabolism in correlation with the level of P-450IA1 and IA2 enzymes in liver microsomal samples. Huge differences were observed in the metabolic profile of caffeine. While the N-7 demethylation (paraxanthine) was the major pathway in man (75% of total dimethylxanthines), the N-3 demethylation (theophylline) was major in monkey (89%), the N-1 demethylation in mouse and rabbit (about 60 and 65% respectively) and the three pathways were equal in rat. Similarly the Σ(dimethylxanthines) / C-8 oxidation ratio varied according to the species.

Four monooxygenase activities known to be supported by cytochrome P-450IA enzymes were measured : methoxy- and ethoxy-resorufin O-dealkylases, phenacetin O-deethylase and acetanilide 4-hydroxylation. All these P-450 dependent activities were correlated with metabolic profile of caffeine.

P-450IA enzymes were immunochemically detected by Western blot technique using polyclonal anti-rat P-450 ß-NF B specific of this sub-family. P-450IA2 enzyme was predominant in the liver of man, rat, rabbit while no polypeptide immunorelated to P-450IA was detected in monkey. Only a minor band corresponding to P-450IA1 was detected in rat and rabbit. All these *in-vitro* data suggest:

- several P-450s are involved in the first oxidative demethylations of caffeine,
-P-450IA2 enzyme is involved in N-3 demethylation of caffeine (paraxanthine formation),
- N-7 demethylation of caffeine (theophylline formation) is carried out by PAH-inducible P-450s other than those of IA family.

MOLECULAR MECHANISM OF SLOW ACETYLATION IN MAN.

Martin Blum, Denis M. Grant, Markus Heim, Anne Demierre and Urs A. Meyer.

Biocenter, University of Basel, 4056 Basel, Switzerland.

The human acetylation polymorphism confers marked interindividual variation in the effect and toxicity of numerous drugs, chemicals and potential carcinogens. Slow acetylators are defective in liver cytosolic arylamine N-acetyltransferase (NAT, EC 2.3.1.5), encoded by the gene NAT2 on chromosome 8. Clinical and toxicological investigations provide evidence that the acetylator phenotype - beside its importance for drug therapy - presents a potential risk factor for several spontaneous and drug induced diseases (bladder cancer). To study the molecular basis of the acetylation defect, we have analyzed NAT at the level of DNA, RNA and protein using the NAT2 gene and an NAT-specific antibody as probes. A genotyping assay based on RFLP analysis identified two common (M1 + M2) and one rare (M3) mutant allele. Cloning and sequencing revealed that these were characterized by single amino acid changes and additional silent base substitutions. Using genotyped liver samples we showed that slow acetylators - although cytosolic NAT2 protein was drastically reduced - contained equal amounts of NAT2-specific mRNA. Functional expression of NAT2 from mutant alleles and chimaeric constructs strongly suggets that this reduction was due to reduced stability of NAT2 protein or altered translatability of mutant mRNA.

EPOXIDE FORMATION DURING THE BIOTRANSFORMATION OF 2-METHYLPROPENE (=ISOBUTENE) IN LIVER TISSUE OF MICE

M. Cornet, W. Sonck, R.J. Laib*, A. Vercruysse and V. Rogiers

Dept. of Toxicology, Vrije Universiteit Brussel, Laarbeeklaan 103, 1090-Brussel, Belgium and *Institut für Arbeitsphysiologie, Abt. für Toxicologie und Arbeitsmedizin, Ardeystrasse, 67, 4600 Dortmund, FRG.

Until now, no data are available concerning the biotransformation and toxicity of 2-methylpropene, a gaseous substance, widely used in industry (rubber, fuel additives, plastic polymers, adhesives).
In this work, the biotransformation of 2-methylpropene has been studied, using total liver homogenates of mice, supplemented with a NADPH-generating system. In analogy to other alkenes, 2-methylpropene is metabolized to its epoxide 2-methyl-1,2-epoxypropane, as proven by its identification by gas chromatography coupled with mass spectrometry. Its formation is cytochrome P-450 dependent, as shown by experiments in the absence of the NADPH-generating system and in the presence of various concentrations of metyrapone and SKF 525-A, two known inhibitors of the mixed function oxidation system.
A simple quantitative headspace method has been developed for the determination of the epoxide formation by gas chromatography. This formation is never linear in function of time and it reaches a maximum after 20 min. Afterwards it decreases continuously to undetectable levels. This observation can be explained by the immediate action of epoxide hydrolase and glutathione S-transferase, converting the epoxide to 2-methyl-1,2-propanediol and to the glutathione conjugate respectively. The involvement of both enzymes has been demonstrated by the addition of 3,3,3-trichloropropene oxide and indomethacin, inhibitors of epoxide hydrolase and glutathione S-transferase respectively. Both increase the epoxide formation in a significant way. The concentration of the free epoxide, is therefore not only dependent on its formation by cytochrome P-450 dependent mono-oxygenases but also on its further metabolisation by epoxide hydrolase and glutathione S-transferase, both very active in liver tissue.

Acknowledgements
The authors wish to thank Prof. M. Van de Walle, Dept. of Organic Chemistry, Rijksuniversiteit Gent for the synthesis of 2-methyl-1,2-epoxypropane and 2-methyl-1,2-propanediol.
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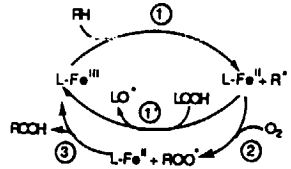
PEROXIDASE-LIKE ACTIVITY OF LIPOXYGENASES : DIFFERENT SUBSTRATE SPECIFICITY OF POTATO 5-LIPOXYGENASE AND SOYBEAN 15-LIPOXYGENASE, AND PARTICULAR AFFINITY OF VITAMIN E DERIVATIVES FOR THE 5-LIPOXYGENASE

C.CUCUROU, J.P. BATTIONI, R. DANIEL and D. MANSUY

Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques (URA 400 CNRS), Université René Descartes, 45 rue des Saints Pères, 75270 Paris Cedex 06, France.

In mammals, a 5-lipoxygenase is involved in the biosynthesis of leukotrienes which have an important role in inflammation and immediate hypersensitivity (1). For mechanistic studies, plant lipoxygenases as soybean lipoxygenase-1 (L-1) and potato 5-lipoxygenase (5-PLO) are particularly interesting, the former as a well-known reference in this field, the latter as a possible model closer to the mammalian 5-lipoxygenase. Only very few compounds, related to fatty acids with a 1,4-cis,cis pentadiene unit are known to act as substrates for the complete dioxygenation cycle of lipoxygenases (L) (scheme steps 1,2,3). However, it has been recently shown that several compounds, which are able to reduce the non-heminic active Fe(III) state of L-1 into its Fe(II) state, act as substrates for a peroxidase-like activity (scheme steps 1 and 1')(2,3).



Scheme: Catalytic cycle for the peroxidase-like activity of lipoxygenase (steps 1 and 1', RH = phenidone for instance in the case of L-1; LOOH = 13-HPOD = 13-hydroperoxyoctadecadienoic acid) and for the dioxygenase activity of lipoxygenase (steps 1, 2 and 3 ; RH = linoleic acid).

We have compared the ability of 5-PLO and L-1 to catalyze the oxidation of various compounds by 13-HPOD, the hydroperoxide derived from linoleic acid dioxygenation by L-1. This study shows that 5-PLO has such a peroxidase-like activity and that L-1 and 5-PLO exhibit very different substrate specificities, phenidone and BW755C being specific to L-1, vitamin E being quite specific to 5-PLO.

As the combined dioxygenase and peroxidase-like activities of lipoxygenases result in the cooxidation of fatty acid and another substrate by O_2, lipoxygenases could participate in the oxidative biotransformation of some xenobiotics.

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STRUCTURAL AND FUNCTIONAL ANALYSIS OF TWO CLOSELY RELATED RABBIT ARYLAMINE N-ACETYLTRANSFERASES.

Daniel D'Orazio, Markus Probst, Martin Blum and U. A. Meyer.

Department of Pharmacology, Biocenter of the Universtity, 4056 Basel, Switzerland.

The New Zealand White rabbit provides a widely used animal model for the human acetylation polymorphism, which confers marked interindividual variation in the effect and toxicity of numerous drugs, chemicals and potential carcinogens. The in vivo and in vitro patterns of arylamine N-acetylation and the predisposition to toxicity in genetically slow acetylator rabbits closely resemble those in slow acetylator humans. Rabbits therefore can be used to study the biochemical basis of the acetylation defect as well as to investigate questions related to tissue-specificity and possible physiological roles of arylamine N-acetyltransferase (NAT; EC 2.3.1.5).

Using a cDNA probe encoding the polymorphic enzyme protein we have isolated two NAT genes (*NAT1*, *NAT2*) from rabbit genomic DNA, and shown that a deletion of *NAT2* accounts for the observed polymorphism. Molecular analysis of the genes revealed that they consist of small noncoding upstream exons and single large coding exons of 870bp. Functional expression of the two genes in monkey kidney COS-1 cells gave rise to two cytosolic enzymes with markedly different kinetic characteristics. NAT1 preferentially metabolized simple arylamine substrates such as p-aminobenzoic acid whereas NAT2 showed high affinities for substrates containing bulky side groups (e. g. sulfamethazine).

While NAT2 is known to play an important role in drug metabolism, NAT1 had never been detected immunologically in liver cytosol before. We have studied the distribution of the two enzymes in various tissues of the rabbit. In Western and Northern type experiments NAT2 was present exclusively in liver and duodenum, while NAT1 was not detectable with these methods at all. The pronounced kinetic differences between the two enzymes, however, allowed a far more sensitive detection and identification of enzyme activity using HPLC analysis of enzyme assays. NAT2 enzyme activity was found to be restricted to liver and duodenum, consistant with its role in drug metabolism, while NAT1 activity was found at low level in all tissues analyzed.

Sequence analysis of *NAT1* and *NAT2* showed that they were highly homologous to each other at both the DNA and protein level, resulting in 13 amino acid changes and a slight shift in electrophoretic mobility of the two proteins on SDS PAGE. We have used these structural similarities as a model system to study structure-function relationships of NAT1 and NAT2. Chimaeric genes were constructed in which the 13 amino acids were sequentially exchanged. Chimaeric genes were inserted into the eukaryotic expression vector p91023(B) and transiently transfected into monkey kidney COS-1 cells. Kinetic analysis of expressed proteins as well as immunoreaction on Western blots revealed that one amino acid change was sufficient to switch enzyme specificity.

QUANTITATIVE STRUCTURE-METABOLISM RELATIONSHIPS OF ESTERASE-CATALYZED HYDROLYSIS OF NICOTINIC ACID ESTERS

A. Durrer, G.N. Wernly-Chung, B. Walther, N. El Tayar and B. Testa

Ecole de Pharmacie, pl. du Château, 1005 Lausanne, Switzerland

Nicotinic acid is a compound of considerable biological importance as a vitamin and a hypolipemic, vasodilating and fibrinolytic drug. In our laboratory, a number of nicotinate esters featuring various alkyl, arylalkyl and aryl substituents have been synthesized [1] as potential prodrugs of nicotinic acid undergoing activation through chemical and/or enzymic hydrolysis. We measured their half-lives of chemical hydrolysis at 37° C and pH 7.4 [2] as well as the kinetic parameters of enzymic hydrolysis (K_M and V_{max}) in several biological media such as rat liver and brain subcellular fractions, purified hog liver carboxylesterase, and human plasma.

This study reports some equations correlating enzymic hydrolysis data with physicochemical parameters such as lipophilicity ($\log k_w^0$) [1,3], molar refractivity (MR) [4], steric descriptors [5], or chemical shifts in ^{13}C- NMR [2] with the aim of gaining some insight into the mechanisms influencing ester binding and catalysis in several tissues.

Hydrophobic interactions were found to govern the binding of nicotinate esters to rat brain and liver esterases. They also play a role, albeit less pronounced, in the binding to human plasma and purified hog liver carboxylesterase. In contrast, no good correlations were found with V_{max}, although hydrophobic interactions, steric hindrance and electronic features seemed to play a role.

Globally, the results fail to document tissue-selective ester hydrolysis.

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CATALYTIC SITE AND MEMBRANE TOPOLOGY OF RAT LIVER MICROSOMAL EPOXIDE HYDROLASE

S.ELSBERG, F.OESCH, H.-D.ZELLER, AND H.THOMAS*, INSTITUTE OF TOXICOLOGY, OBERE ZAHLBACHERSTR.67, D-6500 MAINZ, FRG AND *CIBA-GEIGY AG, CENTRAL TOXICOLOGY UNIT, CH-4002 BASEL, SWITZERLAND

MICROSOMAL EPOXIDE HYDROLASE (mEH_b; E.C.3.3.2.3) CATALYZES THE HYDROLYSIS OF PHYSIOLOGICAL AND XENOBIOTIC EPOXIDES TO MORE POLAR AND BETTER EXCRETABLE VICINAL DIHYDRODIOLS. INVESTIGATING THE REACTION MECHANISM OF THE ENZYME, PURIFIED RAT LIVER mEH_b WAS CHEMICALLY MODIFIED WITH THE HISTIDINE SPECIFIC REAGENT 2-BROMO-4´-NITROACETOPHENONE (BrNAc) CAUSING A TIME AND CONCENTRATION DEPENDENT INACTIVATION. THE COMPETETIVE INHIBITOR VALPROMIDE (VPM, 2mM) PREVENTED THE MODIFICATION, AND ABOUT 70% OF THE ENZYME ACTIVITY WAS RETAINED. INCORPORATION OF ^3H-LABELLED BrNAc INDICATED 1 CATALYTIC SITE HISTIDINE RESIDUE PER PROTOMER. CHYMOTRYPTIC DIGESTION OF MODIFIED ^3H-LABELLED AND OF VPM-PROTECTED ENZYME GENERATED 3 DIFFERENT PEPTIDE FRACTIONS UPON BIOGEL P6 COLUMN CHROMATOGRAPHY. MEDIUM SIZE PEPTIDES WERE PURIFIED BY REVERSED PHASE HPLC (SPHERISORB ODS I+II). RADIOLABELLED PEPTIDES DIFFERENT FROM THOSE OBTAINED FROM VPM-PROTECTED ENZYME WERE SEQUENCED (APPLIED BIOSYSTEMS), AND TWO OF THE DETERMINED AMINO ACID SEQUENCES, LEU-ILE-SER-TYR AND GLU-ARG-GLY CORRESPONDED TO POSITIONS 420-423 AND 427-429 OF THE cDNA-DERIVED AMINO ACID SEQUENCE OF mEH_b [PORTER, T.D. ET AL. (1986) ARCH. BIOCHEM. BIOPHYS.248, 121-129]. THESE RESULTS INDICATE THAT THE PROPOSED CATALYTIC SITE HISTIDINE [DuBOIS, G.C. ET AL. (1978) J. BIOL. CHEM. 253, 2932-2939]. MAY BE IDENTICAL WITH HISTIDINE 431 WHICH IS CLOSE TO THE C-TERMINAL END OF THE ENZYME. THE LATTER IS SUGGESTED TO BE LOCATED OUTSIDE THE MICROSOMAL MEMBRANE AND DIRECTED TOWARDS THE CYTOSOL. TO VERIFY THE LOCATION OF THE C-TERMINUS, SECTIONS OF THE AMINO ACID SEQUENCE OF mEH_b WHICH WERE NOT INCORPORATED INTO THE MICROSOMAL MEMBRANE WERE IDENTIFIED. MICROSOMES FROM STYRENE OXIDE-INDUCED MALE WISTAR RATS WERE DIGESTED USING PROTEASE V8. THE GENERATED PEPTIDES WERE SEPARATED FROM THE MICROSOMES BY ULTRACENRTIFUGATION, AND FURTHER PURIFIED BY IMMUNOAFFINITY CHROMATOGRAPHY USING HIGHLY SPECIFIC ANTIBODIES AGAINST CYTOSOLIC EPITOPES OF mEH_b AS LIGANDS. BOUND PEPTIDES WERE ELUTED, SUBSEQUENTLY PURIFIED BY HPLC AND SEQUENCED. BY COMPARING THE OBTAINED PEPTIDE SEQUENCES WITH THE cDNA-DERIVED AMINO ACID SEQUENCE OF mEH_b ENZYME SECTIONS LOCATED OUTSIDE THE MICROSOMAL MEMBRANE COULD BE IDENTIFIED.

INFLUENCE OF ENVIRONMENTAL FACTORS ON [redacted]HIONE S-TRANSFERASE IN ERYTHROCYTES.

Chris T.A. Evelo and Peter Th. Henderson

Department of Occupational & Environmental Health and To[redacted]
University of Limburg, PO Box 616, 6200 MD Maastricht, The Netherlands.

Glutathione S-transferase activity in human erythrocytes is known to decrease after *in vitro* exposure to various electrophilic compounds. Indications that loss of GST activity may also occur *in vivo* were first found by Kilpikari *et al* [1] for rubber workers. In three separate studies we found indications that decrease in GST activity and in blood GSH concentrations after electrophilic exposure may indeed occur.

- In a preliminary study GST and GSH were determined in blood samples from 2 workers creosoting wood. Both GST and GSH values were markedly lower after a week of work than before that week (after an exposure free period) and returned to normal values after the weekend. Exposure to aromatic electrophiles for these workers was also indicated by increased DNA-adduct levels determined by P_{32} postlabeling methods.
- As part of a prospective study in the Dutch flower bulb culture towards the effects of the soil fumigant 1,3-dichloropropene on applicators, blood GST and GSH values were determined before and after the season [2]. Both GST and GSH were significantly lowered after the season (respective significance levels: $p=0.002$ and $p=0.02$ by Wilcoxon matched-pair analysis).
- GST activity and GSH concentrations in blood from 21 smokers were not significantly different from the the values found for 21 non-smokers [3]. On the other hand the urinary thioether excretion was increased in smokers, and the concentration of GSTρ as determined by ELISA was about doubled compared to the values in non-smokers.

The above results indicate that both blood GST and GSH values may change after exposure to electrophiles. Such changes may have consequences for the capacity of the glutathione conjugating system. Furthermore, determination of such changes may be a valuable monitoring tool in occupational toxicology. The special nature of the changes in smokers found points towards an adaptation by increased GST synthesis during erthrocyte proliferation.

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MULTIPLICITY OF HUMAN P-450 IIC GENES : GENE CLONING DATA AND PHARMACOLOGICAL SIGNIFICANCE

Cécile GED and Philippe BEAUNE

INSERM U75, Faculté de Médecine Necker, PARIS, FRANCE.

Human cytochrome P-450 IIC were first characterized as isozymes responsible for the polymorphic oxidation of S-mephenytoin (1) and are major isozymes in human liver (2). The polymorphism is known as genetically determined (3) but the primary defect had not yet been identified at the protein level (4),(5) and analyses at the cDNA level revealed a complex multigene family (6). In this paper, we are presenting gene cloning data and their pharmacological significance.

Seventeen genomic clones have been isolated from a human genomic librairy in EMBL 4 phage, in Pr. F. Galibert's laboratory (Hôpital Saint-Louis, Paris). Genomic structure, restriction maps and sequencing data from these clones are developped. Using oligonucleotide probes derived from IIC8 and IIC10 cDNAs, the clones are classified in two groups. The first group is composed of P-450 IIC8 related genes and the second group gathers P-450 IIC10 related genes. The first exons from IIC8 genomic clones are identical to the original cDNA. On the opposite, exons from IIC10 related genomic clones differ from IIC10 cDNA. A few single base substitutions are observed simultaneously in five different genomic clones without any alteration in the reading frame. The significance of these P-450 IIC10 related genes is adressed at the transcriptionnal level, at the gene level, and from a pharmacological point of view.

As a conclusion, this work highlights new aspects concerning the relationship between P-450 IIC related genes heterogeneity and S-mephenytoin hydroxylase genetic polymorphism in human.

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CATALYTIC PROPERTIES OF AMINOPYRINE-N-DEMETHYLASE IN RAT LIVER MICROSOMES.

Konstantin E. Gerasimov, and Ilya B. Tsyrlov

Institute of Clinical and Experimental Medicine, USSR Academy of Medical Sciences, Siberian Division, 630117 Novosibirsk, USSR

The catalytic parameters of liver microsomes of rats induced with phenobarbital (PB) and aminopyrine (AP) have been studied in a mutual depletion system. AP-N-demethylation reaction has been inhibited with metyrapone in equimolar to cytochrome P-450 concentrations. The following parameters were obtained: PB-induction - Vmax=10.9 nmoles HCHO/min per 1 mg of protein, Et=8.2 nmoles per 1 mg of protein, TN=1.7 per min. AP-induction - Vmax=9.95, E_t=7.2, TN=1.9. Untreated animals - Vmax=5.5, E_t=4.6, TN=1.25. The obtained results allowed us to conclude that AP-N-demethylation rate increase after PB-treatment is caused more by increase in E_t content and after AP-treatment by TN-increase. The catalytic properties of AP-N-demethylase in rats after PB-treatment has been also studied in vivo by estimation of 4-aminoantipyrine (AAP) excretion in 3 hours urine sample after AP injection. The quantities of AAP excreted with urine in untreated animals fluctuated insignificantly staying at the average level of 23.0 μg. The visible increase of AAP excretion was observed after PB-treatment (80 mg/kg, i.p., 4 days). The maximal excretion rate was registrated on the second day after the last PB injection. The quantity of excreted AAP exceeded the control values by 5 times (116.6 μg vs 23.0 μg). Further with PB-induction effects weakining the differences between induced and untreated animals were smoothing over. It should be emphasized without fail that at the peak of PB induction the strong correlation was observed between the yields of two coupled reaction products formaldehyde in microsomes and AAP in urine of rats. With reference to that substrate it allows us to extrapolate validly the data obtained with urine analysis to estimation of liver monooxygenases state. That has been confirmed by us during AP-test helding among the patients suffering from cholestasis. Preliminary results (considerable decrease in AAP excretion in sick patients related to healthy group) allow us to estimate AP-test as perspective in diagnostics of disturbtions in liver detoxification function in a number of pathological states.

cDNA CLONING AND PARTIAL AMINO ACID SEQUENCE OF RAT LIVER DIHYDRODIOLDEHYDROGENASE

M.Hagen, M.Arandt, H.D.Zeller, F.Oesch, H.Thomas

Institut für Toxikologie, Mainz, W. Germany

Rat liver dihydrodioldehydrogenase (DDH)(E.C.1.3.1.20) is a 34,000 Dalton phase I drug metabolizing enzyme which has been shown to decrease the mutagenicity of different polycyclic aromatic hydrocarbons in the Ames Test. So far, only the biochemical properties of this enzyme have been the subject of several investigations.

A commercial lambda phage gt11 expression library was screened with a monospecific polyclonal antibody against purified DDH and four positive clones containing cDNA inserts ranging in size from 1.2-2.3kb were identified.

An EcoRI restriction fragment of clone no.5 comprising 600bp was subcloned into pUC_{19} and sequenced.The identity of this clone as being part of the cDNA corresponding to the gene encoding for DDH was verified by comparison of the nucleotide sequence of this clone to the amino acid sequence data obtained from tryptic peptides of the purified enzyme.

Since the N-terminus of DDH is known to be blocked, information about the amino acid sequence of this protein could only be obtained from enzymatically generated peptides. Tryptic digestion of purified rat liver DDH and subsequent chromatography of the resulting peptides on RP-HPLC (Vydac C_{18}-column/ 0-70% acetonitrile gradient/ 0.1% TFA) yielded 10 different peptides. These peptides were subjected to gas phase sequencing and a total of 65 amino acids was determined. These amino acids account for about 20% of the total amino acid sequence of DDH.

Now a well defined cDNA probe is available for further screens and for the subsequent isolation and characterization of the full-length cDNA clone for DDH.

The partial cDNA sequence and the peptide sequences will be presented.

CHARACTERIZATION AND IMMUNOINHIBITION OF O-DEALKYLATIONS OF THE NEW CYTOCHROME P450 SUBSTRATES 7-ALKOXYQUINOLINES USING AN END POINT METHOD

B.Hahnemann[1], F.Feil[1], M.Tegtmeier[1], W.Legrum[1], K.J.Netter[1] and O.Pelkonen[2]

[1]Department of Pharmacology and Toxicology, Philipps-University Marburg, Lahnberge, D-3550 Marburg, F.R.G.
[2]Department of Pharmacology and Toxicology, University of Oulu, Kajaanintie 52 D, SF-90220 Oulu, Finland

We introduce an end point method to measure the O-dealkylations by the cytochrome P450 (P450) system of a series of 7-alkoxyquinolines (methoxy- to hexoxy- and benzyloxy-quinoline). The 7-alkoxyquinolines represent structural analogues to 7-alkoxycoumarins and we wanted to investigate whether a nitrogen in the aromatic ring system contributed to metabolic specificity similar to that observed with derivatives of phenoxazones. The fluorescence of the product 7-hydroxyquinoline (7-HOQ), can be measured by a slightly modified 7-ethoxycoumarin assay of Aitio (Anal Biochem 85, 488-491, 1978). The excitation and emission wavelengths are 370 nm and 500 nm, respectively.

To elucidate the effect of the nitrogen atom in the aromatic ring system on the substrate specificity of P450 isozymes we used liver microsomes from male C57BL/6N mice treated with, phenobarbital (PB), 3-methylcholanthrene (3-MC) and pyrazole (PL). The highest turnover number (nmol 7-HOQ/nmol P-450 * min) were obtained with 7-ethoxyquinoline as a substrate (Control: 45; PB: 63; 3-MC: 79; PL: 120). The lowest rates were detected with pentoxy- and hexoxyquinoline (Control: 7.0 and 9.5, respectively). This result shows similarity to the metabolism with coumarin derivatives, where the ethoxy derivative also shows the highest rate. The decreasing rate of metabolism corresponding to the increasing length of the site chain could be explained by an increasing lipophilicity of these substrates or by the steric hindrance at the active site.

Immunoinhibition studies with 7-ethoxyquinoline as a substrate and with several monoclonal (MAB) (including 1-7-1 raised against the major 3-MC inducible P450 isozyme) and polyclonal antibodies (including anti-COH raised against the pyrazole-induced mouse P450 isozyme) as inhibitors, revealed generally weak inhibitions. With control microsomes the MAB 1-7-1 led to the highest inhibition with about 65% remaining activity, while the strongest inhibition (about 45%) could be shown using microsomes of PB pretreated mice with anti-COH. On the basis of these findings it seems possible that P450Coh and P450IA2 both participate in the O-deethylation of 7-ethoxyquinoline.

The 7-alkoxyquinolines are metabolized by the P-450 system and the fluorescence of the product 7-hydroxyquinoline is easily detectable, also the quinolines are metabolized with higher turnover numbers than the corresponding alkoxycoumarins.

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Evidence for the involvement of cytochrome P-450IIIA in the oxidative demethylation of tamoxifen in human liver microsomes.

F. Jacolot, *F. Berthou*, L Simon, P. Beaune* and C. Riché

Laboratoires de biochimie et pharmacologie Faculté de médecine-BP-815-29285-Brest (France); *: INSERM U-75, Hôpital Necker, Paris (France)

The antiestrogen tamoxifen (Tam or Nolvadex, ICI) - trans-Z-1-[4-[2-dimethylamino)-ethoxyl-phenyl] 1,2-diphenyl-1-buten- is widely used in treatment of hormone-dependent breast cancer. The drug is extensively metabolized by cytochrome P-450 dependent hepatic mixed function oxidase in man, yielding mainly the N-desmethyl metabolite (DMT).

This study has been carried out to determine the P-450 enzyme involved in the N-oxidative demethylation of Tam in microsomal samples from 25 human livers (23 adults, 2 children). This metabolic step was inhibited by carbon monoxide up to 85%. Tam was demethylated into DMT with an apparent Km of about 100 µM ; rates varied between 43 and 441 pmol/min/mg microsomal protein.

These metabolic rates were strongly correlated with 6ß-hydroxylation of testosterone (TST) : $r = 0.80$; $p < 0.001$, $n = 25$, activity known to be associated with P-450 IIIA enzyme. To further assess whether or not the Tam demethylation pathway is catalyzed by the same P-450, the inhibitory effect of TST on this reaction was determined. The competitive inhibition had an apparent Ki of about 100 µM. Furthermore the P-450 IIIA content of liver microsomal samples, measured by Western blot technique using a monoclonal P-450 NF(nifedipine) antibody, was strongly correlated with DMT formation : $r = 0.86$; $p < 0.001$, $n = 25$.

All these in-vitro observations establish that a P-450 enzyme of the IIIA sub-family is involved in the oxidative demethylation of tamoxifen in human liver. Accordingly, the known inducers (steroids or macrolide antibiotics) and inhibitors (erythromycin, cyclosporine, nifedipine) of this P-450 may complicate tamoxifen therapy.

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KINETIC STUDIES OF CYTOCHROME P450IID IN NON HUMAN PRIMATES.

E.JACQZ-AIGRAIN, U.A ZANGER*, H.MATHIEU.

Pharmacologie Clinique, Hôpital ROBERT-DEBRE, Paris, FRANCE.
*Pharmacology, Biocenter of the University, Basel, SWITZERLAND.

Genetic polymorphisms in drug metabolism are relevant in therapeutics and in the developement of drugs, but are difficult to investigate in humans. Animal models of predicting value would provided avantages over studies in man for the screening of new drugs early in their developement, in order to determine a possible kinkage with known genetically controled routes of metabolism. Cytochrome P450IID6 exhibits a polymorphic activity under genetic control in man and is involved in the metabolism of a increasing number of drugs. Phenotyping studies, using specific substrates as debrisoquine and dextromethorphan identified 5-10% of poor metabolizers in caucasian populations.

We reported interindividual variations of debrisoquine metabolism in non human primates (macaca fascicularis). In order to characterize P450IID function, we conducted kinetic studies in liver microsomes from extensive metabolizer animals using known substrates of P450IID6 in man: debrisoquine, dextromethorphan and bufuralol. In the NADPH/O2 mediated reaction, the apparent Michaelis Menten constants (Km) for the three substrates were similar to those of the high affinity component of enzyme activity reported in humans. The CuOOH mediated (+)-bufuralol 1'hydroxylation was also determined because it is exclusively catalyzed by P450IID6 in man. The Km of the reaction was 5.5µM (for a human value of 7.3 µM) but in contrast to human results, an additional low affinity component of activity was detected (Km=875 µM).
Various drugs were screened for their possible inhibitory effect on dextromethorphan O-demethylation (n=6) or bufuralol 1'hydroxylation (n=8). The drugs known to be competitive inhibitors of P450IID in man (including debrisoquine, sparteine, flecanide and quinidine) were inhibitors in monkey microsomes. In the presence of dextromethorphan, the Ki for debrisoquine was 58 µM and for quinidine it was 1.4 µM. In contrast, drugs known not to modify P450IID activity in man (including mephenytoine, phenobarbital and phenacetine) had no effect in monkey microsomes.

Kinetics of prototype substrates and substrate specificity of P450IID subfamilly were investigated in non human primates. Enzymatic similarities between humans and non human primates were demonstrated and suggest that this animal species could be a model for human P450IID polymorphism.

EXPRESSION AND ACTIVITY OF CYTOCHROME P450IID IN DEVELOPING HUMAN LIVER.

E.JACOZ-AIGRAIN, T.CRESTEIL*

Pharmacologie Clinique, Hôpital ROBERT-DEBRE, PARIS
*Inserm U75, CHU NECKER, PARIS

Cytochrome P450IID is involved in the metabolism of various xenobiotics and is subjected to a polymorphic activity under genetic control. Phenotyping studies, using specific substrates as debrisoquine or dextromethorphan identified 5-10% of poor metabolizers in caucasian populations. The aims of this study were to investigate the expression and the activity of P450IID in fetal and neonatal human livers.

The immunochemical detection of P450IID and the hepatic content in mRNA coding for the P450IID were examined in 50 fetuses (42 were under and 8 were over the gestational age of 30 weeks), and in 31 neonates (8 were under and 23 were over 24h of life). A detectable level of protein was found in 5/50 fetuses and 23/31 neonates, reaching 30 to 50% of the adult value. The mRna concentration was lower in fetal and neonatal than in adult samples. A positive correlation was found between the mRNA concentration and the specific protein content.
P450IID activity in hepatic microsomes was studied by measuring the O-demethylation of dextromethorphan to dextrorphan in 19 fetal and 3 neonatal samples. Total P450 content was 0.077 ± 0.038nmol/mgP in fetal microsomes and 0.134 ± 0.053nmol/mgP in neonatal microsomes. Dextromethorphan (500µM) and microsomes (total P450 content: 0.225 = 0.120nmol/incubation) were incubated in the presence of a NADPH/O2 regenerating system. Dextrorphan was undetectable in 12/19 fetuses. It was 0.28 ± 0.17ng/mgP/h in the 7 positive fetuses and 4.2 ± 3.4ng/mgP/h in neonates.A positive correlation was found between dextrorphan formation and P450IID content. In contrast, 3-methoxymorphinan, a minor metabolite in adults, was detectable in all the fetal samples (2.05 = 1.69nmol/mgP/h) and in neonatal samples (1.81 ± 1.6nmol/mgP/h). No correlation was found between 3-methoxymorphinan formation and P450IID content, or between the formation of dextrorphan and 3-methoxymorphinan.Our results demonstrate that expression and activity of P450IID develops during late pregnancy and the perinatal period. The metabolic profile of dextromethorphan in developing human livers was different from the profile obtained in adult human livers studied in our laboratory. This comfirms that enzymatic activities develop independantly and raises questions concerning the regulation of gene expression during ontogenesis.

RAT LIVER SULFOTRANSFERASES INVOLVED IN THE CONJUGATION OF HYDROXYLATED PHENANTHRENES, CHRYSENES AND PICENES

A. Johannes, A. Schmoldt, A. Seidel*, H.R.Glatt*, F. Oesch*.

Institute for Legal Medicine, University of Hamburg, Butenfeld 34, 2000 Hamburg 54, FRG, *Institute of Toxicology, University of Mainz, Obere Zahlbacher Str.67, 6500 Mainz, FRG.

Purified cytosolic rat liver sulfotransferases (STs) were tested for their activity towards hydroxylated polycyclic aromatic hydrocarbons (PAH).

Two phenolsulfotransferases (PSTs) and the hydroxysteroid ST 2 were incubated with some hydroxy-PAHs (1-,2-,3-,4-, and 9-OH-phenanthrene, 1-,2-, 3-, 4-, 5-, and 6-OH-chrysene, 1-, 2-,3-, 4-, 5-, 6-, and 13-OH-picene) or its dihydrodiols in the presence of ^{35}S-labeled PAPS.
The hydroxylated compounds were no substrates of the hydroxysteroid ST whereas most of them were extensively sulfated by both PSTs. 1-, 2-, and 9-OH-phenanthrene were by far the best substrates (up to 30 nmoles/min mg proteine). They were conjugated at a 2-5 times higher rate by PST II than by PST I.
Considerable higher ratios of PST II- to PST I-activity were found with 1-, 3-, and 6-OH-chrysene. 2-, 4-, and 5-OH-chrysene were sulfoconjugated only to a very low extent.
For the sulfation of 1-OH-phenanthrene and 1-OH-chrysene by PST II an app. k_m of approx. 12 µmol/l and 0,6 µmol/l, respectively, had been noted.
In comparison to the phenols, the conjugation rates of *trans*-dihydroxy-dihydrophenanthrenes and -chrysenes by the PSTs were very low (traces-600 pmoles/min mg proteine).
A sulfation of the hydroxypicenes or the dihydrodiols was not detectable.

In conclusion, the two PSTs involved in the sulfoconjugation of hydroxyphenanthrenes and -chrysenes showed considerable differences in enzyme activity as well as in regioselectivity.

NAD(P)H:QUINONE REDUCTASE: SAME ENZYMATIC FORM PRESENT IN MURINE LIVER AND SKIN, AND HUMAN KERATINOCYTES.

Frank K.Jugert, Bernd Bonnekoh and Hans F.Merk

Department of Dermatology, University of Cologne, Joseph-Stelzmann-Str.9, 5000 Köln 41, West-Germany

The toxicity of quinones - including certain chemotherapeutic agents such as doxorubicin - have been related to the enzymatic or nonenzymatic formation of the corresponding semiquinones and their subsequent reaction with molecular oxygen yielding superoxide anion radicals by spontaneous regenerating of the quinones [H.Wefers et al., FEBS 169 (1984) 63-66]. This semiquinone redox cycling is prevented by the NAD(P)H:quinone reductase (NQR: EC 1.6.99.2) because it mediates a 2-electron reduction which results in the formation of hydroquinones instead of semiquinones. Interestingly inducers of this enzyme such as butylated hydroxytoluene protect against the severe ulceration of accidental infiltration of doxorubicin into the area around the intravenous infusion. NQR belongs to the aromatic hydrocarbon responsive [Ah] battery. This gene battery includes Cyp 1a1 (Cytochrome $P_1$450), Cyp 1a2 (Cytochrome $P_3$450) and Nmo-1 (NAD(P)H:quinone reductase) [D.D.Peterson et al., PNAS 86 (1989) 6699-6703]. In the skin cytochrome $P_1$450 dependent activity is about 1-5 % compared to its activity in the liver, whereas NQR has the same activity in skin and liver [W.H.Khan et al., BBRC 146 (1987) 126-133]. The human gene of the NQR is localized on chromosome 16 and has been cloned recently as well as the gene of the murine liver NQR [A.K.Jaiswal et al., JBC 263 (1988) 13572-13579].
The purpose of this study is to characterize the cutaneous NQR-activity by known inhibitors of different reductases and to compare it with the murine skin and liver NQR-activity.
NQR was determined in the cytoplasma of murine skin, liver, and human keratinocytes using 2,6-dichlorophenol-indophenol (DCPIP) as substrate [L.Ernster, Meth.Enzym. 10 (1967) 309-317, A.M.Benson et al., PNAS 77 (1980) 5216-5220]. The enzyme activity was expressed as nmol DCPIP reduced/ min/ mg protein.
In order to characterize this enzyme induction by polycyclic hydrocarbones and inhibition with several known inhibitors of dihydrodiol dehydrogenase, aldo-keto and carbonyl reductase activities [K.Post et al., 11thEuropean Workshop on Drug Metabolism (A)., 1988, 1.46], were determined.
The basal NQR-activity was similar in murine skin and liver, however the induction with polycyclic hydrocarbons was stronger in the liver. There was a similar pattern of inhibition of the basal and induced activity in all tissues so far investigated. Pyrazole, progesterone and phenobarbital did not inhibit, however rutin and indomethacin inhibited dose dependently. The most potent inhibitor was dicoumarol.
There was an identical pattern of NQR-inhibition in human keratinocytes, murine skin and liver. This suggests that the NQR present in liver and skin, and in mouse and human is a similar NQR-isoenzyme.

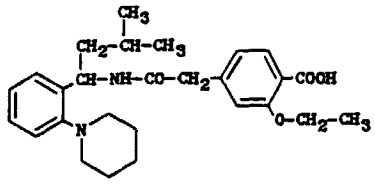
P 1.22

BIOTRANSFORMATION OF THE ENANTIOMERS OF AG-EE 388 ZW IN RAT

S. Kaschke, J. Schmid, A. Greischel

Dept. of Pharmacokinetics & Metabolism, Dr. Karl Thomae GmbH, D-7950 Biberach/Riss, F.R.G.

AG-EE 388 ZW, 2-Ethoxy-4-[N-(1-(2-piperidino-phenyl)-3-methyl-1-butyl)-aminocarbonylmethyl]-benzoic acid is a racemic anti-diabetic drug candidate.



After i.v.-administration of 0.2 mg/kg of the racemate and the enantiomers as ^{14}C-labelled drugs the biliary excretion and the biotransformation were investigated in male and female rats.

With bile between 88 - 97 % of the administered dose are eliminated within 6 hours.
The initial elimination rate of total radioactivity is highest in the (S)-enantiomer in male rats and lowest in the (R)-enantiomers in female rats.
No parent compound is eliminated with bile, however, the enantiomers are different in their metabolic pattern in bile of male rats. Administering the (S)-enantiomer we find two main metabolites N2 and N7, administering the (R)-enantiomer a third metabolite N3 appears to about 25 %.

The structures are:
N2: Ring opening of the piperidine-ring yielding a 1-amino-5 carboxylic acid.
N3: Deethylation of the $-O-CH_2-CH_3$ group and consecutive glucuronidation.
N7: Glucuronidation at the carboxylic acid of the intact molecule.

In bile of female rats we find only the metabolites N2 and N7 in different ratios, depending on the enantiomer.

For the interpretation of the toxicological studies (performed with the racemate) it appears important that the metabolic pattern of the racemate is between the composition of the enantiomers, so we can exclude that one enantiomer influences the metabolism of the other.

P 1.23

TURNOVER NUMBER OF CYTOCHROME P-450IIB1 IN LIVER MICROSOMES OF DIFFERENT AGE RATS TREATED WITH PHENOBARBITAL-TYPE INDUCERS.

Oleg G. Khatsenko, Lyudmila F. Gulyaeva, Vladimir M. Mishin and Vyacheslav V. Lyakhovich.

Institute of Clinical and Experimental Medicine, Academy of Medical Sciences of the USSR, 630117 Novosibirsk, USSR

Metabolism of androstenedione (AD) in rat liver microsomes has been investigated. Both neonatal (1-4 weeks) and adult (8 weeks and more) have been induced with PB-type inducers such as phenobarbital (PB), perfluorodecalin, 2,4,6-triphenyl-1,3-dioxan and Aroclor 1254.

It has been shown that treatment of rats by these inducers considerably increases (by 10-30 times) the AD 16β-hydroxylation rate in microsomes compared to control. In addition, small increase of 16α- and 6β-hydroxylation rate has been detected.

From PB-liver microsomes of adult rats an electrophoretically homogeneous P-450IIB1 was isolated which at high rate oxidised AD in 16β position. ELISA method with monospecific antibodies to this P-450 form was applied to measure its content in microsomes. The P-450IIB1 content was measured to be 3-4 times smaller in newborns than in adults. The same antibodies almost completely inhibited production of 16β-hydroxymetabolite. This allows to conclude that P-450IIB1 is responsible for this reaction in treated animals of different age, the turnover number being at least 2-3 times higher in newborns than in adults.

These data confirm earlier obtained by us results on the high turnover number of cytochrome P-450IA1 in methylcholanthrene induced neonatal rats. This phenomenon is not related to the content of reductase in newborns since their reductase to P-450 ratio does not considerably differ from that in adults. Further studies on the regulation of catalytic activity of neonatal P-450's is needed.

METABOLISM OF ACETANILIDES BY CYTOCHROME P450; A GENERAL MECHANISM INVOLVING ONE-ELECTRON OXIDATION STEPS.

Johan M. te Koppele, Luc Koymans, Gabriëlle Donné-Op den Kelder and Nico P.E. Vermeulen.

Department of Pharmacochemistry, Faculty of Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

Nowadays, the mechanism whereby cytochrome P450 (P450) metabolizes xenobiotic substrates is thought to occur by two sequential one-electron oxidation steps rather than by a single transfer of the reactive oxygen from P450 to the substrate (1). After the first one-electron step (abstraction of a H-atom, i.e. $H^+ + e^-$) a substrate radical is formed which can (before or after radical rearrangements) combine with the activated oxygen species of P450 (supposedly a heme bound $OH^\bullet$ radical) or undergo alternative reactions typical for radical species (1). The present study was performed to investigate whether P450-mediated metabolism of *para*-substituted-acetanilides can be satisfactory explained with such a general mechanism of one-electron steps and radical rearrangements.

Metabolism of paracetamol (*para*-hydroxy-acetanilid) can occur by initial H-atom abstraction at the N- or O-atom (route I and II resp.). In a previous theoretical study it was shown that H-abstraction at the O-atom was about 30 kcal/mole more favorable than H-abstraction at the N-atom (2). All paracetamol metabolites could be explained by initial H-abstraction from the O-atom, rearrangements to carbon centered radicals at C-1, C-3 or C-5, followed by recombination with the heme bound $OH^\bullet$ radical or by a second H-abstraction yielding *N*-acetyl-*para*-benzoquinonimine).

This mechanism was also found to hold true for the *para*-ethoxy-derivative of paracetamol (q.e. phenacetin). Then, initial H-abstraction can occur both at the N-atom (route I; allowing for radical rearrangements to C-1, C-4 and C-6), and the α-ethoxy carbon (route II, lacking radical rearrangements). The formation of paracetamol as metabolite of phenacetin can be explained by route II. Formation of *N*-acetyl-*para*-benzoquinonimine, *N*-hydroxy- and 2-hydroxy-phenacetin all suit metabolism of phenacetin via route I and are consistent with currently available experimental data on metabolism of ^{14}C- and ^{15}O-labeled phenacetin (3).

Finally, metabolism of *para*-chloro-acetanilid was investigated. Abstraction of the hydrogen atom can only occur at the N-atom (route I) inasmuch as the *para*-chloro substituent doesn't allow H-abstraction via route II. Consistently, oxidative metabolism of *para*-chloro-acetanilid resulted in exactly the metabolites predicted from the H-abstraction and rearrangement model: *N*-acetyl-*para*-benzoquinonimine, *N*-hydroxy- and 2-hydroxy-*para*-chloro-acetanilid.

In conclusion, cytochrome P450-mediated metabolism of *para*-substituted acetanilides can be satisfactory explained by initial H-atom abstraction (at the N-atom or at the *para*-substituent, if the latter has the appropriate characteristics), followed by a second H-abstraction or intramolecular radical rearrangements with subsequent recombination with a hydroxyl radical.

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MODELING THE ACTIVE SITE OF CYTOCHROME P450-DEBRISOQUINE

Johan M. te Koppele, Luc Koymans, Nico P.E. Vermeulen and Gabriëlle Donné-Op den Kelder.

Department of Pharmacochemistry, Faculty of Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

As a consequence of the genetic polymorphism of cytochrome P450-debrisoquine (P450-IID6) the metabolism of debrisoquine and some 20 other drugs is impaired in 1-30% of various populations (1). Inasmuch as P450-IID6 has the remarkable feature that it only metabolizes substrates with a basic nitrogen atom (in contrast to other P450 isoenzymes) an active site model of P450-IID6 has been described in which the presence of a carboxylate group at the active site was assumed (2). The distance between the oxidation site and the basic N-atom was reported to be 5 Å in contrast to the 7 Å proposed in another model (3). The '5Å-model' is unable to fit the rigid '7Å-substrate' dextrometorphan, while the '7Å-model' does not accommodate '5Å-substrates' like debrisoquine and sparteine. The present study was undertaken to combine and extend both models so that contradictions are eliminated.

First, support for the hypothesis that a carboxylate group could exist at the active site was obtained from a comparison of primary amino acid sequences of 53 cytochromes P450. Interestingly, all P450 isoenzymes of the IID class (human, mouse, rat) contain a carboxylic acid moiety (Glu-441) that is absent in all other P450 isoenzymes.

Second, with molecular modeling techniques the interaction of over 20 substrates (5Å as well as 7Å substrates) with the postulated carboxylate group was investigated. Oxidation sites of all substrates were fitted in such a way that the adjacent atoms had a planar conformation. Stereoselectivity in the metabolism was also taken into account. As expected, the N-atom of the 5Å-substrates could not be fitted at the 7Å-position, while the N-atom of the 7Å-substrates could not be matched with the 5Å-position. Nevertheless, the localization of basic N-atoms at a distance of 5Å or 7Å from the oxidation site could be accommodated in one model by postulating a 'bidentate interaction' of the carboxylic acid group. Thus, the N-atom of a 5Å-substrate interacts with the oxygen atom of the carboxylate closest to the oxidation site; the N···O interaction of a 7Å-substrate then occurs at the oxygen atom of the carboxylate that is furthest away from the oxidation site.

Evaluation of this postulated model revealed a new common feature of all (5Å and 7Å) substrates for P450-IID6. Near the oxidation site (in the planar part of the molecule) all substrates have negative molecular electrostatic potentials (< -10 kcal/mole). Thus, an electrostatic interaction between (a π-system, N- or O-atom of) the substrate and a complementary electrostatic field at the protein might be important.

In conclusion, the present study supported the presence of a carboxylate group at the active site of P450-IID6 (possibly from Glu-441) and lead to an active site model that satisfactory described the metabolism of 5Å as well as 7Å substrates by P450-IID6. The predictive value of the active site model is currently under study with a selected set of compounds that was not used to derive the model and for which the metabolism by P450-IID6 is known.

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ACETONE HYDROXYLASE ACTIVITY IN MALE WISTAR RATS TREATED WITH DIFFERENT CYTOCHROME P-450 INDUCERS

Nina E. Kozlovskaya, Valentine A. Vavilin, Vladimir M. Mishin, Vyacheslav V. Lyakhovich

Institute of Clinical and Experimental Medicine, Academy of Medical Sciences of the USSR, 630117 Novosibirsk, USSR

Acetone hydroxylation to 1-hydroxyacetone (acetol) is a P-450 dependent reaction and therefore its activity can vary upon the influence of monooxygenase system inducers.

Acetone hydroxylase activity has been studied in liver microsomes from control rats as well as rats treated with phenobarbitone, Aroclor 1254, acetone and isoniazid. Liver microsomes from control, acetone- and isoniazid-treated rats catalyzed acetone hydroxylation at rates of 1.91± 0.43; 8.16± 0.96; 3.96± 0.52 nm formed acetol/min/mg of protein, respectively. For acetone Km,app of this reaction was equal to 33mM in microsomes from acetone-treated rats. Liver microsomes from rats induced with phenobarbitone and Aroclor 1254 did not catalyze acetone hydroxylation.

To estimate the in vivo acetone hydroxylase activity we determined the acetol concentration in blood serum of control rats as well as acetone- and isoniazid-induced animals possessing an increased acetone hydroxylase activity in liver microsomes. Only in the blood serum of acetone-treated rats acetol at the concentration of 442± 58 nm/ml has been discovered whereas control and isoniazid-treated rats had not detectable acetol in the serum.

The obtained data thus suggest that acetone hydroxylation in rats is a specific reaction for P-450IIE1 since inducers of this cytochrome increase acetone hydroxylase activity in liver microsomes. Apparently, in the cases when sufficient amount of acetone - substrate of this reaction is available in organism, the acetol level in the blood serum can indicate P-450IIE1 activity in vivo.

IMMUNOINHIBITION STUDIES AND THE EFFECT OF METYRAPONE ON HEPATIC COUMARIN 7-HYDROXYLASE AND TESTOSTERONE 15α-HYDROXYLASE ACTIVITIES IN MAN AND MOUSE

Jukka Mäenpää[1], Paavo Honkakoski[2], Matti A. Lang[2] and Olavi Pelkonen[1]

[1]Department of Pharmacology and Toxicology, University of Oulu, Kajaanintie 52 D, SF-90220 Oulu, Finland
[2]Department of Pharmacology and Toxicology, University of Kuopio, POB 6, 70211 Kuopio, Finland

In the mouse there are two closely related forms of enzymes catalyzing 15α-hydroxylation of testosterone belonging to P450IIA subfamily, which are different in only 11 substitutions in their 494 amino acid residues. The type I enzyme catalyzes effectively 15α-hydroxylation of testosterone. The type II enzyme has a much lower testosterone hydroxylation activity, but it has been shown to catalyze 7-hydroxylation of coumarin. Coumarin 7-hydroxylase (COH) activity varies a lot in different human liver samples, but it is higher than in uninduced mouse liver microsomes. Pyrazole induces both COH and testosterone 15α-hydroxylase (15αOH) in mice. It has been shown that the antibody against mouse P450Coh inhibits clearly COH activity in both human and mouse liver microsomes.

The purpose of this study was to investigate COH and 15αOH in human liver microsomes and compare them with corresponding enzymes in pyrazole induced DBA/2N mouse liver microsomes. Human liver contained low levels of 15αOH (about 10-30 pmol/min/mg protein) when compared with the mouse liver microsomes (about 800 pmol/min/mg protein). Other testosterone metabolites were measured, too. Human liver microsomes had a high 6β-hydroxylase activity (about 2500 pmol/min/mg protein), while mouse liver microsomes had a high 7α-hydroxylase activity (about 600 pmol/min/mg protein), which also is a member of the P450IIA subfamily.

The antibody against P450Coh inhibited efficiently mouse liver 15αOH and had an effect on 7α-hydroxylase, too, but it didn't inhibit human 15αOH or other testosterone oxidating enzymes. Metyrapone inhibited efficiently and selectively mouse 15αOH (about 2% remaining activity at 500μM), when compared with 7α- (22%), 6β- (41%) and some other hydroxylations which were not so efficiently affected. In human liver microsomes almost all testosterone hydroxylations were strongly and equally inhibited (e.g. 15αOH 9% and 6βOH 4% remaining activity). Metyrapone strongly inhibited COH in mouse liver microsomes, but had no effect on COH activity in human liver microsomes.

On the basis of available evidence human and mouse P450Coh isozymes seem to be orthologous enzymes whereas the present results indicate that the human $P450_{15\alpha}$ is different from the mouse $P450_{15\alpha}$.

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OXYDATION RATE OF OXODIPINE BY RAT AND HUMAN MICROSOMES CHARACTERISATION OF THE CYT. P450 ISOENZYME INVOLVED

C. MAS-CHAMBERLIN*, M. CHABIN*, J.P. FLINOIS**, P. BEAUNE**, A. DUFOUR*

* Department of Biopharmaceutics, Laboratoires DELAGRANGE, 1 avenue Pierre Brossolette, 91380 CHILLY MAZARIN, FRANCE
** INSERM-U75, CHU Necker, 156 rue de Vaugirard, 75730 PARIS, FRANCE

Oxodipine is a novel dihydropyridine calcium channel blocker under development, with antihypertensive properties.
In order to obtain early information on the metabolic capacities in man of the gastrointestinal mucosa and of the liver towards an oxodipine oral formulation, the rate of biotransformation for this drug in these tissues was determined in vitro with microsomal preparations from human biopsies. This approach was first validated in rats, for which the in vitro rate of oxydation was shown to reflect the in vivo time course of oxodipine in plasma of rats.
The apparent Km were similar in rat and in man for the liver as well as for intestine : 40 - 60 uM, values close to other reported kinetic constants of dihydropyridines. The inter-individual variability was looked for in man with 13 liver biopsies, a variation coefficient of 40 % was found for the maximum rate (Vm) of liver biotransformation towards oxodipine at concentrations of 80 uM and 200 uM.
For the human intestine, the Vm was 10 times lower than for the liver leading to the assumption that the intestinal barrier would play a minor role in the metabolism of Oxodipine.
The identification of the isoenzyme responsible for the oxydation of oxodipine was conducted : competitive inhibition with erythromycin (Ki 100 uM) and immunoinhibition (up to 90 %) with purified anti-P450 III A gave evidence that this isoenzyme was mainly involved in the oxydation of oxodipine.
This finding, together with the knowledge of local concentration of P450 III A present in the intestine, allowed us to evaluate the capability of intestinal metabolism as 1/700 of that of the liver.
The advantage of in vitro data obtained with human tissues and the predicitve capacity in vivo of these data, will be discussed.

COMPARATIVE STUDIES ON CARBONYL REDUCTASES FROM PROKARYONTS AND MAMMALS.

Edmund Maser, Udo Oppermann, Karl J. Netter.

Department of Pharmacology and Toxicology, Philipps-University Marburg, School of Medicine, Lahnberge, 3550 Marburg, FRG.

In contrast to extensive studies on enzymes catalyzing oxidative drug metabolism, enzymes mediating reductive biotransformation of xenobiotics have obtained less attention. This is specially true for carbonyl reductases which are responsible for the reduction of aldehydes and ketones to the corresponding alcohols. They are found in various mammalian tissues and located in the endoplasmic reticulum or cytoplasm of the cell. However, their specific physiological roles remain to be determined.
Using metyrapone (MP), the diagnostic cytochrome P-450 inhibitor as a substrate for carbonyl reduction (Maser, 1989; Maser and Netter 1989), we found carbonyl reductase activity in cytosol and microsomes of rat, mouse and guinea pig liver and kidney. While the cytosolic enzymes were characterized as ketone reductases, the microsomal enzymes turned out to be aldehyde reductases with respect to inhibitor classification of carbonyl reductases.
The ubiquitous distribution and multiplicity of carbonyl reductases suggest a more general role of these enzymes in the metabolism of carbonyl compounds of both exogenous and endogenous origin. Cofactor requirement and molecular weight of the purified MP reductase from mouse liver microsomes and the ability of dihydrotestosterone to competitively inhibit MP reduction suggest this enzyme to be related to 3α-OH steroid dehydrogenase. Consequently we tested 3α-OH steroid dehydrogenase from Pseudomonas testosteroni, a steroid inducible bacterium, for its ability to reduce metyrapone and found a much higher activity compared to that of mammalian liver microsomes.
For estimating immunological homologies between these enzymes polyclonal antibodies were raised against the microsomal mouse liver MP reductase. Applying the immunoblot technique the antibodies crossreacted only with one single protein band of liver microsomal fractions from all three animal species corresponding to the mouse liver microsomal reductase in the 34 kDa molecular weight region. Thus, besides showing similarities in inhibitor sensitivity and specificity to MP as substrate, the liver microsomal enzymes seem to be structurally related. However, no crossreaction was detected with any protein of liver cytoplasm or kidney microsomes and cytoplasm from the three animal species, indicating the absence of common antigenic determinants.
Surprisingly, the antibodies also crossreacted specifically with the Pseudomonas protein in the 28 kDa molecular weight region, which represents its 3α-OH steroid dehydrogenase activity as well as its metyrapone reductase activity as shown by activity staining after native gel electrophoresis.
From these results we conclude that the liver microsomal MP reductase is an enzyme capable not only of reducing carbonyl compounds but also of participating in androgen inactivation. Moreover, the common antigenic determinants of MP reducing proteins of mammalian liver microsomes and prokaryonts suggest that carbonyl reducing enzymes must have been highly conserved during evolution.
The principle of biological conservation requires that the system fills an important role in normal cell physiology, which, in case of carbonyl reductases, probably is the metabolism of steroids.

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MECHANISTIC ASPECTS OF THE OXIDIZING CAPACITY OF HEMOGLOBIN TOWARDS PHENOTHIAZINE DRUGS

N.J. de Mol, P.R. Kelder, L.H.M. Janssen

Utrecht University, Faculty of Pharmacy, Dept. Pharmaceutical Chemistry, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands

The oxidative capacity of hemoglobin (Hb) has drawn attention because of certain similarities between reactive Hb forms and reactive forms of cytochrome P-450. We studied the oxidative capacity of Hb towards phenothiazine drugs (PHs). Hb is only able to oxidise PHs after reaction with H_2O_2. Reactions between PHs and methemoglobin (metHb) have been monitored spectrophotometrically, while also O_2 and H_2O_2 consumption have been assayed. Based on the various experiments a reaction cycle is proposed: Firstly reaction of metHb with H_2O_2 leads to the formation of a transient ferryl Hb species with a porphyrine radical cation. This species is analogous to compound I observed in peroxidases. This transient species is most likely the reactive Hb form in the oxidation of PHs. An electron is transferred from PH to the compound I analogue leading to the formation of PH radical cations ($PH^{+\cdot}$). In the absence of PHs the compound I analogue is fastly transformed into a ferryl species with a protein radical structure, the radical being located on a tyrosine residue near the heme[1]. This protein radical Hb species has a life-time of several minutes and is oxidised to a peroxy radical by consumption of oxygen. Ultimately a relatively stable ferryl Hb species is formed which decays in approximately 1 h to (modified) metHb. Reaction of metHb with H_2O_2 denatures Hb and induces protein-heme cross links. Reaction with PHs partly protects against denaturation and cross-linking. Part of the formed $PH^{+\cdot}$ reacts with Hb and binds covalently to it, another part of $PH^{+\cdot}$ decays mainly into its sulfoxide. The reaction rate of PHs with metHb plus H_2O_2 is in agreement with its electron donating ability: PHs with a 2-substituent having low σ_p value react faster.

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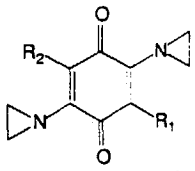
REDOX CYCLING AND REACTIVE OXYGEN INTERMEDIATES PRODUCTION OF POTENTIAL ANTITUMOUR BISAZIRIDINYL QUINONES

N.J. de Mol, K.J. Lusthof, L.H.M. Janssen

Utrecht University, Faculty of Pharmacy, Dept. Pharmaceutical Chemistry, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands



3,6-Disubstituted-2,5-bis(1-aziridinyl-)1,4-benzoquinones (BABQs) are bioreductively activated to DNA alkylating species. Like many other bioreductively activated antitumour quinones e.g. mitomycin C, adriamycin, daunomycin BABQs are able to undergo redox cycling, which possibly leads to the formation of reactive oxygen species like superoxide anion ($O_2^{-\cdot}$), H_2O_2 and $OH^{\cdot}$, for this the equilibrium of eq. 1 is important.

$$Q + O_2^{-\cdot} \rightleftharpoons Q^{-\cdot} + O_2 \qquad \text{eq.1}$$

(Q and $Q^{-\cdot}$ are quinone and semiquinone respectively).
BABQ was reduced with xanthine oxidase (XO) with xanthine (X) as the electron donor. BABQs are efficiently reduced by X/XO, the reduction rate correlated with the reduction potential and steric parameters for the 3,6-substituents. During BABQ reduction with X/XO the production of $O_2^{-\cdot}$, H_2O_2 as well as that of $OH^{\cdot}$ was assayed. Depending on the redox potential of the BABQ derivatives the equilibrium of eq.1 appeared to be shifted to the right. The decrease of $O_2^{-\cdot}$ correlated well with the observed decrease in $OH^{\cdot}$ production. Also the amount of single strand breaks in plasmid DNA decreased in the presence of BABQ, in contrast to the observations with the classical antitumour quinone adriamycin. The results can be explained on basis of the relatively high redox potential of BABQs compared to classical antitumour quinones. For the $OH^{\cdot}$ production and $OH^{\cdot}$ induction DNA single strand breaks, $O_2^{-\cdot}$ is needed in a Fenton reaction sequence. Therefore, decreased $O_2^{-\cdot}$ also implies decrease of the harmful $OH^{\cdot}$ radicals.

MEASURE OF DRUG METABOLIZING ENZYME ACTIVITIES ON FETAL RAT LIVER CELLS DURING CULTURE

L. NEGRO, C. COLLIGNON, A. TODARO, P. KREMERS

Laboratoire de Chimie médicale, Institut de Pathologie,
Bâtiment B35, Université de Liège
B-4000 Sart Tilman par Liège 1, Belgium

Primary fetal hepatocytes constitute an attractive alternative model for the study of drug metabolism and toxicity.

An appropriate treatment with glucocorticoids maintains these cells in culture for several weeks without loss of enzymatic activities. Monooxygenase are inducible in these cells that are also able to express cytochrome P-450's characteristics of adult rat liver.

To record the effects of a drug on the cells during a long period of time it is interesting to measure enzymatic activities on ongoing culture without interrupting the culture by harvesting the cells.

Several assays have been adapted in order to measure monooxygenase activities directly on cells in culture. The substrates are dissolved in the culture medium, they are transformed by the cells and the metabolites excreted in the medium. The medium collected for quantification of the metabolites is replaced by a fresh one and the culture continued. The metabolites are hydrolysed by incubation with a mixture of glucuronidase/sulfatase and quantified according to the usual methods.

Several monooxygenase activities including benzopyrene hydroxylase, ethoxyresorufin deethylase, pentoxyresorufin dealkylase, ethoxycoumarin deethylase, aldrin epoxydase, testosterone metabolism have been measured by this way. The data (enzymatic activities) were compared to those obtained on harvested and homogenized cells. The excellent correlations found demonstrate the validity of this method to approach problems of chronic toxicology as well as regulation studies concerning the drug metabolizing enzymes.

CHARACTERIZATION OF PIG LIVER PURIFIED CYTOCHROME P-450 ISOENZYMES FOR OCHRATOXIN A METABOLISM STUDIES.

Thierry Oster, Zaid Jayyosi, Edmond E.. Creppy*, Hajar Souhaili El Amri and Anne-Marie Batt.

Centre du Médicament, URA CNRS 597, Faculté des Sciences Pharmaceutiques et Biologiques, 54000 Nancy, France, and
*UER des Sciences Pharmaceutiques et Biologiques, 33076 Bordeaux, France.

Ochratoxin A (OTA) is the major ochratoxin metabolite produced by different species of the genera *Aspergillus* and *Penicillium*.
Ochratoxin is a mycotoxin responsible for severe devastations in pig and poultry breadings. Moreover, it has been shown to be involved in human Balkan endemic nephropathy.
The metabolic fate of OTA in rat and rabbit liver microsomes yields unchanged OTA, OT alpha and oxidated metabolites, such as (4-R)-hydroxy-OTA and (4-S)-hydroxy-OTA. Castagnero et al (1989) reported that OTA-4-hydroxylase was inducible in rat liver by phenobarbital as well as by 3-methylcholanthrene. They also postulated a co-segregation of genes regulating OTA and debrisoquine 4-hydroxylation.
As the pig is a target animal species for OTA toxicity and as it is known that the liver monooxygenases represent the main system involved in OTA metabolism, we characterised four pig liver cytochromes P-450 and we further studied the OTA in vitro metabolism in microsomes and in reconstituted monooxygenase system.
Three constitutive pig liver cytochromes P-450 isoenzymes were able to oxidize OTA, one quantitatively minor isoenzyme yielding a new metabolite.
None of the isoenzymes involved in OTA metabolism cross reacted with polyclonal antibodies raised against rat liver P-450 IA. These isoenzymes cross reacted with anti rat liver P-450 IIB polyclonal antibodies.

Anti P-450 IIB	++	++	+
Anti P-450 IA	-	-	-
4S-OHOTA	+	-	-
4R-OHOTA	+	+	+
"New" Metabolite	-	-	+

EFFECT OF AROMATASE INHIBITORS ON ESTROGEN 2-HYDROXYLASE IN RAT LIVER MICROSOMES *IN VITRO*

H. S. Purba, E.J. King, P. Richert & A.S. Bhatnagar.

Research Department, Pharmaceutical Division, CIBA-GEIGY Ltd., CH-4002 Basel, Switzerland.

Inhibition of estrogen production by aromatase inhibitors is now an established drug treatment for breast cancer in postmenopausal women. However, it is possible to lower circulating estrogens not only by inhibiting their synthesis but also by stimulating their rate of degradation. On the other hand, inhibition of estrogen metabolising enzymes would sustain estrogen levels. Estrogens are mainly metabolised to 2-hydroxyestrogens in the liver by the enzyme estrogen 2-hydroxylase. Thus the aim of this study was to examine the effects of the aromatase inhibitors, aminoglutethimide, 4-hydroxyandrostenedione and CGS 16949A on estrogen 2-hydroxylase in rat liver microsomes *in vitro* using estradiol as a substrate. Ketoconazole, an inhibitor of androgen biosynthesis and a potent inhibitor of estrogen 2-hydroxylase was used as a reference drug.

Washed liver microsomes were produced from female Sprague Dawley rats by differential centrifugation. Protein content was determined by the method of Lowry *et al.* Estradiol 2-hydroxylation was measured by a product isolation method using HPLC and by a tritiated water method employing [2-^{3}H]-estradiol as a substrate. Incubations containing estradiol (20 µM), inhibitor (0-750 µM), ascorbic acid (1 mM), NADPH (0.6 mM), microsomal protein (0.5-1.0 mg) and phosphate buffer (1/15 M; pH 7.4) were performed in a final volume of 1.0 mL at 37° C. The reaction was stopped after 8 min with the addition of HCl (6 M; 100 µL). The incubate was eluted on a disposable C_{18} column with methanol, and the eluate injected onto a C_{18} reversed-phase HPLC column. 2-Hydroxyestradiol was quantified using mestranol as an internal standard. For the tritiated water method, [2-^{3}H]-estradiol (0.5 µCi) was added to unlabelled estradiol (20 µM) and incubated as above. The reaction was stopped with the addition of dichloromethane (10 mL). Following extraction, an aliquot of the aqueous phase was counted for radioactivity. For the determination of enzyme kinetic parameters, incubations were performed containing varying concentrations of estradiol and a fixed concentration of the test compound. Kinetic parameters were derived from a Lineweaver-Burk plot. Using the tritiated water method, the Km for estrogen 2-hydroxylase was 4.3 µM using estradiol as a substrate with a Vmax of 11.89 nmol/h/mg.

	HPLC method	Tritiated water method		
	IC_{50} (µM)	IC_{50} (µM)	Ki (µM)	Type of Inhibition
Ketoconazole	2.2	4.1	2.6	Non-competitive
4-Hydroxyandrostenedione	98	88.7	71.7	Competitive
CGS 16949A	110	123.5	113.6	Non-competitive
Aminoglutethimide	908	775.7	958	Non-competitive

The IC_{50} values for the reference drug ketoconazole and the aromatase inhibitors were similar using both methods. Compared to ketoconazole, all three aromatase inhibitors were relatively weak inhibitors of estrogen 2-hydroxylase *in vitro*. This suggests that these compounds will not affect estrogen degradation *in vivo*.

IMMUNOCHEMICAL CHARACTERIZATION OF NAD(P)H:QUINONE OXIDO-REDUCTASE AND CARBONYL REDUCTASE

Barbara Ruepp and Bendicht Wermuth

Chemisches Zentrallabor, Inselspital Bern, 3010 Bern

Quinones and structurally related compounds exert toxic effects in biological systems. These compounds are reduced by single-electron transfer to the reactive semiquinones which readily donate the electron to oxygen, yielding superoxide anions and other cytotoxic, active oxygen species. Alternatively, the quinones may be reduced by two-electron reduction to the relatively stable hydroquinones that are conjugated with glucuronate or sulfate and excreted. Experimental evidence from a number of laboratories indicates that enzymes mediating two-electron reduction of quinones protect the cell against quinone-induced cytotoxicity by competing with the single-electron reduction pathways.

Two enzymes that catalyze the two-electron reduction of quinones are present in human tissues: NAD(P)H:quinone oxidoreductase (NQOR, EC 1.6.99.2), formerly designated as DT-diaphorase, and carbonyl reductase (CR, EC 1.1.1.184). Both enzymes catalyze the reduction of essentially the same quinone substrates, which has hampered studies on their role in the detoxication of quinones based on enzyme activity. Recently, sequence analysis of the two enzymes has shown that they are structurally not related, thus offering the possibility of immunochemical discrimination of the two proteins. Antibodies against NQOR and CR were raised in rabbits, and IgGs were isolated by ammonium sulfate precipitation and DEAE-cellulose chromatography. No cross-reactivity between the antibodies and the two reductases was detectable by double immunodiffusion and on Western blots. For quantitative determinations of the two enzymes an ELISA was developed. Serially diluted antigen of known (standard) and unknown concentration was incubated with a constant amount of antibody and an aliquot transferred to microtiter plates coated with the corresponding antigen (2 ug/ml). Bound antibodies were quantitated spectrophotometrically using horseradish peroxidase coupled to protein A. A marked increase in sensitivity was observed when organomercurials (p-mercuribenzoate, ethylmercurithiosalicylate) were included in the incubation buffer. In this case, the detection limit for both enzymes was approximately 5 ng.

MONOCLONAL ANTIBODIES DIAGNOSTIC FOR INDIVIDUAL MEMBERS OF THE CYTOCHROME P450IV GENE FAMILY

C. Savoy*, C.R. Wolf°, M. Villermain*, H. Thomas* and F. Waechter*
*Central Toxicology Unit, CIBA-GEIGY Limited, CH-4002 Basel, Switzerland, and °Imperial Cancer Research Fund, Department of Biochemistry, Edinburgh EH8 9XD, Scotland.

Introduction Clofibrate-inducible lauric acid ω- and (ω-1)-hydroxylation was shown to reside predominantly on distinct proteins of the cytochrome P450IV gene family. In the rat, at least three members of this gene family have been characterized by cDNA (P450IVA1 and P450IVA3) and/or genomic nucleotide sequencing (P450IVA1 and P450IVA2) (1-3). Cytochrome P450IVA1 shares about 65% sequence similarity with P450IVA2 and A3, while the coding DNA sequences are 97% similar between P450IVA2 and A3. Although polyclonal antibodies against purified P450IVA1 provided evidence for at least two forms of P450IV proteins, they did not differenciate between P450IVA1 (51.5 kDa) and the in vitro translation product of P450IVA3 mRNA (52,0 kDa). The apparent molecular weight of P450IVA2 upon SDS-PAGE has not been assigned yet.

Results and Discussion Hybridomas were formed from myeloma cells and spleen cells derived from BALB/c mice immunized with i3I and fIIp, two different cytochrome P450IV preparations, from clofibrate-treated male SD rat liver. Hybridoma clone clo1 derived from the i3I antigen produced a monoclonal antibody (MAb) which was diagnostic for the P450IVA1 protein as deduced by Western blot analysis from the known apparent P450IVA1 molecular weight and induction profile in liver and kidney of Nafenopin-treated male SD rats as compared with controls. From the clo-hybridomas obtained after immunization with the fIIp antigen, 12 independent MAb-producing clones were selected. Ascites were raised and immunoglobulins purified for the clones clo1 (IgG2b), clo4 (IgG2), clo5 (IgG1) and clo6 (IgG1). Immunoglobulins were characterized by isoelectric focusing, crossreactivity with the antigens used for immunization, ELISA and Western blotting as well as for their inhibitory potency for the lauric acid 11- and 12-hydroxylation of microsomes from Nafenopin-treated rat liver. As a result, clone clo1 specifically recognized the P450IVA1 protein, while clone clo6 specifically recognized two proteins of MW 51.5 kDa and 52.0 kDa represented by the antigen fIIp. From the lack of crossreactivity of clo6 with the antigen i3I it is concluded that the 52 kDa protein represents cytochrome P450IVA3 and the 51.5 kDa band corresponds to the closely related P450IVA2. Clones clo4 and clo5 both recognized all three proteins of the P450IV gene family. Clo5 and clo6 efficiently inhibited the lauric acid 11- and 12-hydroxylation of Nafenopin induced rat liver microsomes by 73.5% and 79.8%, and 74.5% and 78.2%, respectively. Using these MAbs, tissue and sex specific regulation of P450IV expression at the protein and activity level in liver, kidney and lung of SD rats will be discussed.

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HUMAN LIVER MICROSOMAL MONOOXYGENASES ARE UNAFFECTED BY AGE AND GENDER

D.L. SCHMUCKER[a], K.W. WOODHOUSE[b], R.K. WANG[a], M.Mc MANUS[c] and P. KREMERS[d]

[a] Cell Biology and Ageing VAMC San Francisco, USA ; [b] Department of Medicine Royal Victoria Hospital, Newcastle, UK ; [c] Department of Clinical Pharmacology, Flinders University, Adelaide, Australia ; [d] Institut de Pathologie, CHU Liège, Belgium

There is considerable clinical pharmacokinetic evidence which suggests that drug disposition is compromised in the elderly. Studies have implicated alterations in the distribution, reductions of blood flow and/or liver volume, reductions of hepatic phase I metabolism and impairment of renal clearance in this subpopulation.

Most of the experimental evidence demonstrating age and sex dependent shifts in the concentrations and activities of liver microsomal monooxygenases has been accrued in rats. There are no definitive data regarding the status of liver microsomal monooxygenases as a function of age and gender in healthy humans.

Liver microsomes were prepared from 59 (14-89 years of age) male and female organ donors or at laparatomy (Wedge biopsy). The microsomal content in cytochrome P-450 isozymes and in cytochrome P-450 reductase has been measured by immunological methods using specific monoclonal antibodies.

Several enzymatic activities have been determined.

The activity of NADPH cytochrome c reductase per mgr of microsomal protein did not change significantly with age in either sex. The microsomal concentration of immunoprecipitable reductase (expressed as reciprocal titer) declines slightly with age in females. Expression of these values as μg reductase/gr of liver tissue diminished these differences.

The response of cytochrome P-450 (CO binding spectra) to ageing was similar : apparent age and gender differences when expressed per mgr of microsomal protein, no significant differences when expressed per gr of liver.

The concentration of cytochrome P-450 III A2 dit not change or correlate with increasing age in either sex. P-450 II C8 declined in both sexes although the correlation was weak. Analysis of the isozyme/total cytochrome P-450 ratios in both sexes substantiated the absence of significant correlations between age and isozyme content.

The present study provides the most extensive and comprehensive data to date which demonstrate the absence of significant age and/or gender dependent differences in the activities and contents of human liver monooxygenases.

BIOCHEMICAL CHARACTERIZATION OF THE ENZYMATIC FACTOR CONJUGATING MONOHALOGENATED METHANES IN HUMAN ERYTHROCYTES

K. R. Schröder, U. Föst, S. Deutschmann, H. Peter, E. Hallier

Institut für Arbeitsphysiologie an der Universität Dortmund, Abteilung Toxikologie und Arbeitsmedizin.

Previous studies which concentrated on the conversion of monohalogenated methanes by erythrocytes in the presence of glutathione showed a conversion restricted to man. Two subpopulations were found: erythrocytes of 60% of the volunteers converted monohalogenated methanes to S-methylglutathione (conjugators) while 40 % did not (non-conjugators). Glutathione S-transferases were thought to be responsible for this conversion. Inhibition experiments with 1-chloro-2,4-dinitrobenzene and sulfobromophthalein, a specific inhibitor of the minor form of the erythrocyte glutathione S-transferase, supported this theory.

Studies on the conversion of ethylene oxide by human erythrocytes showed the existence of the same two subpopulations as for the conjugation of monohalogenated methanes: conjugators metabolized ethylene oxide while non-conjugators did not. The existence of two subpopulations for the conjugation of ethylene oxide and monohalogenated methanes in the presence of glutathione suggests that these substances are metabolized by the same enzymatic factor. Since epoxides are good substrates for the glutathione S-transferase class Mu, this class may be involved in the metabolism of monohalogenated methanes. This is supported by the fact that tha conjugation of monohalogenated methanes is inhibited by the epoxide trans-stilbene oxide.

In order to investigate the enzyme activity in erythrocytes, glutathione S-transferases were purified by affinity chromatography. However, the eluted transferases did not show the expected activity against monohalogenated methanes. The enzymatic activity responsible for the metabolic turnover of monohalogenated methanes and ethylene oxide in human erythrocytes is therefore possibly not associated with the major form of the erythrocyte glutathione S-transferase. Since glutathione can be substituted by cysteine as cosubstrate in the enzymatic assay, other enzymatic factors must be taken into account.

EXPRESSION OF CYTOCHROMES P-450 HOMOLOGOUS TO THE MAJOR RAT LIVER ISOENZYMES IN HUMAN LIVER MICROSOMES.

H. Thomas, M. Villermain*, U.A. Meyer, C.R. Wolf and F. Waechter*. CIBA-GEIGY Limited and Biocenter of the University (U.A.M.), CH-4002 Basel, Switzerland, and Imperial Cancer Research Fund, Edinburgh, U.K. (C.R.W.).

Trans-species homologies among cytochrome P-450 isoenzymes of rat and 21 human liver samples were investigated by Western blot analysis of microsomes with monoclonal antibodies (MAb) against purified rat liver isoenzymes. While MAb derived from the hybridoma clone b/e4 (diagnostic for P450IIB1/B2) did not recognize any human cytochrome P-450, at least one strong signal indicating an apparent molecular weight of 54 kDa was obtained with MAb d15 which was diagnostic for P450IA1/A2. Weak signals with individually varying intensity were observed using each, the P450IVA1-specific MAb c101, the P450IVA2/A3-selective MAb c106 and MAb c104 which has been shown to recognize all three rat liver P450IVA proteins. MAb k1 (P450IIC6) detected at least two different antigens, the lower molecular weight component of which appeared less intense and occasionally missing. MAbs h7 and h8 (P450II C11) yielded a minimum of three strong signals with significant interindividual variation in the expression of the corresponding antigens. A good correlation was obtained with the microsomal EROD activity (13-173 pmol/min/mg) and the Western blot signals of clone d15. Except for four samples (10-40 pmol/min/mg) no microsomal turnover of PROD could be detected, in contrast to significant cyclophosphamide oxidation at considerably varying levels by all human liver samples.